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Running title: Iodine and LPO in DMBA-induced mammary tumors

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Abstract

Previously, several groups including ours reported that iodine exhibited antiproliferative and apoptotic effects in various cancer cells only if this element is supplemented as molecular iodine, or as iodide to cells that are able to oxidize it by the enzyme thyroperoxidase. In the present work we analyzed the effect of various concentrations of iodine and/or iodide in the dimethylbenz[a]anthracene (DMBA) mammary cancer model in rat. The results show that 0.1% iodine or iodide increases the expression of peroxisome proliferator-activated receptors type gamma (PPARγ), triggering caspase-mediated apoptosis pathways in damaged mammary tissue (DMBA-treated mammary gland) as well as in frank mammary tumors, but not in normal mammary gland. DMBA treatment induces the expression of lactoperoxidase, which participates in the antineoplastic effect of iodide and could be involved in the pro-neoplastic effect of estrogens, increasing the formation of DNA adducts. In conclusion, our results show that supplements of 0.1% molecular iodine/potassium iodide (0.05%/0.05%) exert antineoplastic effects, preventing estrogen-induced DNA-adducts and inducing apoptosis through PPARγ/caspases in pre-cancer and cancerous cells. Since this iodine concentration does not modify the cytology (histology, apoptosis rate) or physiology (triiodothyronine and thyrotropin) of the thyroid gland, we propose that it be considered as an adjuvant treatment for premenopausal mammary cancer.
Introduction

A robust body of information supports the notion that moderately high concentrations of iodine may reduce pathologies in several iodine-uptake tissues like thyroid, mammary gland, intestine, and prostate (Venturi et al. 2000; Aceves & Anguiano 2009). It has been shown that iodide (I\(^-\)) exerts antiproliferative and apoptotic effects in thyrocytes and that the rate-limiting step for this effect is the conversion of I\(^-\) to a more reactive iodine species like molecular iodine (I\(_2\)) catalyzed by thyroperoxidase (TPO). Indeed, Vitale et al. (2000) showed that an excess of potassium iodide (KI) induces apoptosis in cultured thyroid cells, but if TPO activity is blocked with propylthiouracil, the apoptotic effect of KI is eliminated. Moreover, using lung cancer cells (without natural iodine uptake) transfected with the iodide/sodium symporter (NIS) or NIS/TPO, Zhang et al. (2003) observed that only in NIS/TPO-transfected cells did excess KI induce apoptosis, indicating that I\(^-\) from KI needs to be oxidized to have a cytotoxic effect. Data obtained in either in vivo (n-methyl-nitrosourea; MNU) or in vitro (the human mammary cancer cell line MCF-7) models of mammary cancer show that I\(_2\) but not I\(^-\) (KI or NaI) exerts an antineoplastic effect by increasing the expression of peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)), which triggers apoptotic mechanisms that include the Bax-casapase and AIF pathways (Garcia-Solis et al. 2005; Shrivastava et al. 2006; Aceves et al. 2009). Moreover, recently we demonstrated that 6-iodolactone (6-IL), which is generated by the iodination of arachidonic acid, is a specific ligand of PPAR\(\gamma\) (Nuñez-Anita et al. 2009). The finding that I\(^-\) has no effect in these models has been explained by the absence of the same components required in thyrocytes: NIS for uptake of iodide and/or lactoperoxidase (LPO) for its oxidation. NIS is necessary to internalize I\(^-\) in
several tissues (Dohan et al. 2003), whereas LPO, a protein homologue of TPO, oxidizes iodide and binds it to the lactoprotein casein, which is secreted together with free iodide into the milk (Kussendrager & VanHooijdonk 2000). LPO is generally present in mammary gland only during pregnancy and lactation (Dohan et al. 2003), but it has been detected in some mammary cancer models (Anderson et al. 1984). It has been proposed that LPO is involved in mammary carcinogenesis, because of its ability to react in vivo and in vitro with estrogen molecules, oxidizing them to catechol estrogens that bind to DNA and induce mutations (Lovstad 2006; Cavalieri et al. 1997).

The dimethylbenz[a]anthracene (DMBA) model is considered a good model of human mammary cancer, because DMBA is a pro-carcinogen that is metabolically oxidized to a polycyclic aromatic hydrocarbon (PAH), a well-known class of environmental factors that contribute to the etiology of breast cancer in humans (Thompson 2000). Some reports have shown that the DMBA-induced mammary cancer cells express NIS and LPO (Anderson et al. 1984), and that co-administration of Lugol’s solution - a mixture of I₂ and potassium iodide (KI) - with medroxy progesterone acetate significantly decreases the growth of mammary tumors (Funahashi et al. 1996). However, the different chemical forms and doses of iodine have not been analyzed in this model. In the present work we investigate the effects of different combinations of iodine and/or iodide on mammary glands (non-tumoral and tumoral) from control and DMBA-treated rats and the participation of PPARγ and LPO in these effects. Our results confirmed that in this model, the iodide transporters NIS and pendrin (PEN) are present as well as LPO, and that iodine treatment overstimulates all of these components in the tumors. It was also
shown that iodine, at concentrations of 0.1% or more and as either \( \text{I}_2 \) or \( \Gamma \), is necessary to induce the antineoplastic effect and that this effect is accompanied by significant increases in PPAR\( \gamma \) expression and caspase-3 activity. Other important findings are that LPO is involved in protection by iodide and also in the pro-neoplastic 17\( \beta \)-estradiol (E2) stimulation (increasing the level of DNA adducts), since its inhibition with methimazole (MMI) reduced both effects.

**Materials and Methods.**

**Animals**

Virgin female Sprague-Dawley rats were born in a colony maintained at the Instituto de Neurobiología, Universidad Nacional Autónoma de México (UNAM). Animals were kept under regulated temperature conditions (22 ± 1 °C) at 50% humidity on a 12 h:12 h light-dark cycle and permitted *ad libitum* access to food (Purina Certified Rodent Chow) and water. Animals were housed and handled in accordance with UNAM Animal Use Committee Guidelines.

**Iodine supplement**

Virgin five-week-old rats were supplemented with the following preparations: 0.05% molecular iodine (0.05% \( \text{I}_2 \)), 0.05% potassium iodine (0.05% KI), 0.07% \( \text{I}_2 \), 0.05% \( \text{I}_2 \) + 0.05% KI (MIX), 0.1% KI, 0.05% \( \text{I}_2 \) + 0.1% KI (Lugol), 3 \( \mu \)g/ml thyroxine (T4), 0.05% methimazole + 3 \( \mu \)g/ml triiodothyronine (MMI/T\( _3 \)) and MMI/T3 + MIX in drinking water for 22 weeks. Distilled water was used for drinking water and for all solutions. The
concentration of the iodine preparations was confirmed by titration with sodium thiosulfate (Kenkel et al. 1994).

Carcinogen administration and tumor incidence

Two weeks after the iodine treatments began, all rats were anesthetized with a mixture of ketamine and xylazine (Cheminova, México, 30 mg and 6 mg, respectively, per Kg bw), and a single intragastric dose of 20 mg/ml DMBA (Sigma, St. Louis, MO) dissolved in 1 ml of sunflower oil was administered. The control group received 1 ml of sunflower oil alone. Tumor incidence and growth were analyzed for 20 weeks after DMBA administration. Endpoints for data analysis were: time to tumor appearance (tumor latency), percentage of animals that developed tumors (tumor incidence), number of tumors per rat (tumorogenesis), and combined size of tumors at the end of treatment (tumor volume). At the end of the experiment the rats from each group were sacrificed by decapitation. Tumor sizes were measured using a caliper, and volumes were calculated by the ellipsoid formula (Russo & Russo 2000). If the tumors reached the ethically maximal size of 3 cm³, rats were anesthetized with the ketamine/xylazine mixture, and the tumors were surgically removed even before the end of the experiment. Normal or tumoral mammary glands were fixed in 10% neutral buffered formalin or frozen in dry ice and kept at -70°C. Blood was collected to determine circulating levels of total iodine, T3, and TSH.

Thyroid status.
Iodine, $T_3$, and TSH circulating levels. Total iodine was measured by HPLC and $T_3$ levels by the homologous RIA methods previously described (Garcia-Solis et al. 2005). TSH levels were quantified by the ELISA method (Biosource, Camarillo, CA).

Thyroid gland integrity. After 20 weeks of DMBA and/or iodine treatments, formalin-fixed, paraffin-embedded thyroid sections (5 µm) were dyed with the hematoxylin and eosin (H&E) method, and frozen thyroids were used for the apoptosis assay (see below).

RT-PCR.

PPAR$\gamma$, NIS, PEN, and LPO expression were analyzed by quantitative real time PCR (qPCR). Total RNA was obtained using the TRIzol reagent (Life Technologies, Inc., Carlsbad, CA) dissolved in RNAase-free water (50 µL), and stored at -70°C. The extracted RNA (2 µg) was reverse transcribed using oligo-deoxythymidine. In order to eliminate genomic DNA contamination, we carried out the RT assay for each individual sample and as control, we used one tube that contained a sample from a pool of all samples but no transcriptase enzyme (-RT). We ran a standard PCR for each pair of oligos with two individual samples (random) and the -RT control. The sequence detector system Roto-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) was used to perform qPCR with SYBRgreen as a marker for DNA amplification. The reaction was performed with 1 µL of cDNA template and the qPCR supermix-UDG kit (Invitrogen), using 40 cycles of three-step amplification (94°C for 30 s, 55–60°C for 30 s, 72°C for 30 s) and the gene-specific primers listed in Table I. PCR generated only the expected specific amplicon, which was demonstrated in each case by the melting temperature
profile (dissociation curve) and by electrophoresis of 5 µL of the PCR product through a 2% agarose gel containing ethidium bromide in TAE buffer. No PCR products were observed in the absence of template. Gene expression was calculated using the D-cycle threshold (Dct) method and normalized to the content of actin, a non-regulated housekeeping gene. The coefficient of variation for this gene was less than 15% in all RT-PCR assays, indicating that the significant changes observed in the different groups correspond to changes in the experimental genes.

Caspase-3 activity

Caspase-3 activity was measured using a standard colorimetric kit (Sigma). Briefly, frozen mammary glands, tumors, or thyroids were homogenized (1:2) with lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT, 4°C). Samples were centrifuged at 16,000 g for 20 min at 4°C, and supernatants were collected and stored at -70°C. Protein concentrations were determined by the Bradford method (BIO-RAD protein assay, Richmond, CA), and 100 µg of protein was assayed for caspase-3 activity. The substrate was 200 µM DEVD-pNa, which contains the chromophore p-nitroanilide (pNa) linked to a synthetic tetrapeptide DEVD (acetyl-Asp-Glu-Val-Asp). The final cocktail (300 µL) was incubated in assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.01% CHAPS, 5 mM DTT) for 3 h at 37°C with continuous agitation and protected from light in a 96-well plate. Samples were read at 405 nm in an ELISA reader, and caspase-3 activity was expressed as nmol of pNa released/h/mg protein.

Quantification of in situ cell death.
Cell death was detected in formalin-fixed, paraffin-embedded mammary gland or tumor sections using the *in situ* fluorescein/POD cell detection kit (Roche Molecular Biochemicals, Mannheim, Germany), which is based on the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Sections (5µm) were prepared and treated according to the manufacturer’s instructions. Diaminobenzidine was used as the chromogen, and sections were counterstained with hematoxylin. TUNEL-positive cells were identified by a brown stain over the nucleus. Five regions, chosen at random, were analyzed, and labeling indices were expressed as the percentage of labeled cells among at least 500 cells per region.

Estrogen and LPO participation

In a second round of experiments, rats with frank tumors (~ 2 cm²) were treated for two weeks with daily injections of 17β-estradiol (E₂; 0.5 µg/day) and given drinking water with and without the LPO inhibitor methimazole and triiodothyronine only (MMI/T₃) or together with MIX (MMI/T₃ + MIX). Triiodothyronine (T₃) was used to maintain euthyroidism (Alva-Sanchez *et al.* 2009). The pro-neoplastic effect of E₂ and the possible protective effect of iodine (MIX) as well as how these effects were influenced by LPO inhibition (MMI/T₃) were analyzed by the number of DNA adducts, as well as by the change in the tumor growth (size of tumor) after 14 days. E₂, T₃, and MMI were obtained from Sigma.

DNA isolation and ³²P-postlabeling analysis of DNA adducts
Normal and tumoral mammary glands were pulverized in liquid nitrogen and homogenized in a solution of 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 100 mM EDTA, and 0.5% SDS. After RNase and proteinase K treatment, DNA was isolated by phenol-chloroform extraction. The DNA concentration was determined spectrophotometrically at 260 nm. DNA adducts were analyzed by the nuclease P1 enrichment version of the $^{32}\text{P}$-postlabeling assay according to the protocol by Phillips & Arlt (2007). The labeled DNA adducts were separated by thin layer chromatography on PEI-cellulose plates (Machery-Nagel, Düren, Germany) using the following solvents: D1, 1 M sodium phosphate, pH 6.0; D2, 3.6 M lithium formate, 8.5 M urea, pH 3.5; and D3, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. The plates were autoradiographed, and the diagonal radioactive zone was cut out for Cerenkov counting. All samples were run in triplicate.

Statistical analysis

The effects of dietary treatments on mammary cancer incidence were analyzed using 2 X 2 contingency tables and a chi-square test. The effects of treatments on the other variables were analyzed using one-way ANOVA and Tukey’s honest significant difference tests. Values with $p < 0.05$ were considered statistically significant.

Results

Figure 1 and 2 and, table 2 and 3 summarize the chronic effect (22 weeks) of low or moderately high concentrations of iodine, iodide, or both, as well as LPO inhibition (MMI/T$_3$ treatment) on iodine and thyroid hormone status and on the incidence and progression (tumorogenesis and tumor volume) of DMBA-induced tumors. Circulating
levels of total iodide were consistent with the low or moderate ingestion of iodine, and no significant modifications in circulating levels of T3 or TSH were observed (figure 1). Incidence analyses showed that concentrations of 0.1% or higher of either chemical form of iodine were necessary to exert a significant antineoplastic effect (figure 2). The data also showed that inhibition of LPO activity impaired the antineoplastic effect of 0.1% KI and MIX (by 80% and 50%, respectively). The tumorogenesis and tumor size were significantly lower in all iodine-treated animals in comparison with the DMBA control group (table 3).

To analyze the cellular/molecular mechanisms involved in the antineoplastic effect of moderately high concentrations of iodine, we used samples of the MIX group. As shown in figure 3, MIX treatment induced significant increases in PPARγ expression and caspase-3 activity in both damaged (DMBA-treated) mammary gland and frank tumors. These increases correlated with the significant rise in DNA rupture (TUNEL-positive cells) found in these tissues (figure 4).

To explore the effect of iodine supplement on the proteins needed for the uptake and metabolism of iodine, we measured the expression of NIS, PEN, and LPO in normal and tumoral mammary gland using lactating mammary tissue (ten days postpartum, 8 pups) as a positive control. The results showed that normal mammary gland contains low concentrations of NIS, PEN, and LPO. In contrast, in both mammary gland and tumors from DMBA-treated animals all these proteins are overexpressed, and when tumors are treated with MIX this overexpression increases even more (figure 5).
Analyses of thyroid glands showed that only supplements containing the highest concentrations of iodide (0.1% KI and Lugol) exhibited flat thyroid epithelium, but no change in caspase-3 values was observed at any dose (figure 6).

To analyze the possible participation of LPO in the oxidation of iodide and/or in the transformation of E\textsubscript{2} into electrophilic metabolites, a second experiment was performed in animals with frank tumors. In this experiment (figure 7) we analyzed the number of DNA adducts in mammary gland and tumors as well as the change in tumor size after 2 weeks of treatment with E\textsubscript{2}, MIX, and/or MMI/T\textsubscript{3}. E\textsubscript{2} treatment increased the number of DNA adducts and decreased tumor growth. MIX treatments significantly reduced the number of DNA adducts in both DMBA-damaged mammary tissue and tumors, and they prevented the increase in DNA adducts induced by E\textsubscript{2}; the MIX-treated group also showed the maximum reduction in tumor size. MMI/T\textsubscript{3} reduced the formation of DNA adducts in response to E\textsubscript{2} treatment and partially blocked the antineoplastic effect of MIX, as measured by tumor size (MMI/T\textsubscript{3}+MIX +E\textsubscript{2}). The weak antineoplastic effect of MMI/T\textsubscript{3} alone was not significant.

**Discussion**

Here, we demonstrated that in the DMBA mammary cancer model, both iodine and iodide can diminish and delay tumor initiation and incidence, and they decrease tumor size in frank tumors. We also showed that the antineoplastic iodide effect was blocked if LPO activity was inhibited (MMI/T\textsubscript{3}), suggesting that iodide needs to be oxidized to exert...
this effect. In previous reports we demonstrated that 0.05% I\textsubscript{2} is enough to prevent 60 to 70% of the MNU-induced mammary tumors (Garcia-Solis \textit{et al.} 2005); however, in the DMBA model, drinking water containing 0.1% or more iodine or iodide is necessary to observe this effect. It is well established that DMBA is more aggressive than MNU in generating malignant tumors, and the DMBA model is considered to be more similar to breast cancer with respect to the PAH-initiation mechanisms (Al-Dhaheri \textit{et al.} 2008). It is possible that a larger quantity or different types of iodometabolites are necessary to decrease the progression of these DMBA-induced cancers, or that PAHs produce different types of iodine-sensitive tumor cells. In the first case, more than one mechanism or iodine component may be triggered or generated by the iodine/iodide supplement. Several groups have postulated that the antineoplastic I\textsubscript{2} effect could be mediated by direct antioxidant/oxidant action at the mitochondrial level or by the formation of iodolipid intermediates like 6-IL or iodohexadecanal (Dugrillon \textit{et al.} 1994; Pisarev \textit{et al.} 1994; Shrivastava \textit{et al.} 2006; Rosner \textit{et al.} 2010). Our group has demonstrated that 6-IL formation in solid tumors (MNU) or cancerous cells (MCF-7) is associated with elevated concentrations of its precursor arachidonic acid (AA), and that 6-IL formation is independent of peroxidases when the supplement is I\textsubscript{2} (Arroyo-Helguera \textit{et al.} 2006; Arroyo-Helguera \textit{et al.} 2008; Aceves \textit{et al.} 2009). DMBA tumors were previously reported to contain high concentrations of AA and/or its metabolites (i.e. prostaglandins) (Tan \textit{et al.} 1974; Rillema & Mulder 1978).

In relation to molecular mechanisms, our results showed that iodine induces significant expression of PPAR\textgamma in DMBA-treated mammary glands and frank tumors, but not in normal mammary gland, and that this increase correlates with the activation of caspase-3.
and the induction of DNA rupture (TUNEL), suggesting that PPARγ could be a crucial mediator of the antineoplastic iodine effect. These results agree with our previous data showing that the apoptotic effect of iodine or 6-IL is accompanied by the significant induction of PPARγ in both *in vitro* and *in vivo* models (Aceves *et al.* 2009; Nuñez-Anita *et al.* 2009) and that 6-IL is a specific activator ligand of this receptor (Nuñez-Anita *et al.* 2009). Moreover, a recent report shows that pre-treatment with rosiglitazone - a potent agonist of PPARγ - synergizes the anticancer activity of cisplatin and minimizes cisplatin-induced nephrotoxicity by stimulating PPARγ expression in DMBA-induced tumors (Tikoo *et al.* 2009).

Iodine supplement was also accompanied by significant increases in NIS, PEN, and LPO in mammary tumors, which contrast with the null effect in normal mammary gland or with the classical inhibition of NIS and TPO expression observed in normal thyroid gland (Anguiano *et al.* 2007). One possible explanation is that under normal conditions, high doses of iodine decrease the expression of these genes, as part of a mechanism to regulate thyroid function, whereas in cancer situations, the upregulation of these proteins by iodine might be strongly associated with tumoral redifferentiation. A study in thyroid cancer cell lines has shown that high doses of molecular iodine increase the expression of p53, p21, and Bcl-xL, suggesting that iodine might promote the process of cell redifferentiation (Liu *et al.* 2010). These antitumor effects have also been attributed to PPARγ activation. There is evidence that PPARγ agonists promote cell redifferentiation, which is reflected in the upregulation of specific differentiation markers (NIS, TSH receptor, LPO, thyroglobulin) and downregulation of a dedifferentiation marker (CD97).
in thyroid cancer (Park et al. 2005; Aiello et al. 2006). Additional experiments are required to confirm this hypothesis with respect to breast cancer.

Exposure to high iodine concentration is a potential risk for thyroid physiology (leading to hypothyroidism or autoimmune disease) as well as for general health (retinal damage) (Paul et al. 1988). However, careful examination shows that pathological responses occurred at low or moderate iodide intake in patients with underlying or evident thyroid pathology (e.g. Hashimoto’s thyroiditis, history of treatment for Graves’ diseases, etc.), but in normal subjects only with higher doses of iodine or iodide (>20 mg/day) (Robison et al. 1998; Burgui et al. 2001). No damaging effects were reported in either the human or animal studies that used therapeutic I\(_2\) concentrations (3 to 15 mg/day) (Ghent et al. 1993; Kessler et al. 2004; Anguiano et al. 2007; Aceves & Anguiano 2009). In the present study our results showed that the thyroid epithelium response was directly proportional to the iodide concentration: iodide or iodine concentrations of 0.05% or less did not modify the cytoarchitecture (epithelium/lumen ratio), but 0.1% KI or Lugol decreased this ratio with the tissue exhibiting thinning thyroid epithelium and increased luminal volume, which suggested a hypothyroid-like situation. However, after 22 weeks of supplementation with all iodine or iodide concentrations tested, there was no evidence of apoptotic thyrocytes (caspase-3 activity) or changes in T3 or TSH circulating levels, indicating that at these iodine/iodide doses, the thyroid gland was able to make physiological adjustments to maintain the normal thyroid status.
Another important finding in this study is the participation of LPO in the antineoplastic effect of iodide. Previously, it was reported that DMBA tumors contain LPO activity (Anderson et al. 1984); here we demonstrated that this enzyme is expressed and that its inhibition results in a long-term (20 weeks) or short-term (2 weeks) decrease in the antineoplastic effect of iodide. It is well established that LPO is a powerful peroxidase capable of oxidizing iodide even more efficiently than TPO (Davies et al. 2008; Guo et al. 2004). The presence of LPO in pregnant and lactating mammary gland is considered to be an adaptive mechanism to ensure an adequate supply of iodine to the neonate (Memphan 1987). This enzyme has been reported to be present at a very low concentration or even absent in non-lactating mammary gland and in some tumor cell models like MNU tumors or MCF-7 and MDA-MD231 cells (Anguiano et al. 2007; Arroyo-Helguera et al. 2008); however, the present data show that LPO is expressed in precancerous and cancerous, DMBA-treated mammary cells. Furthermore, preliminary data of our laboratory demonstrate the presence of LPO in samples of early and advanced human breast cancer (unpublished data). These findings become particularly relevant since LPO can oxidize natural or synthetic estrogens to catechol estrogens (Lovstad 2006; Cavalieri et al. 1997). The resulting estrogenic quinones have been shown to react with DNA to form mutagenic adducts that can initiate or promote cancer (Cavalieri et al. 1997). To follow this line of thinking, we asked whether LPO, in the presence of moderately high levels of 17β-estradiol (E2) and/or iodine (MIX), could be contributing to tumor growth and/or to the number of DNA adducts in frank tumors. Our data showed several novel results: first, mammary glands from DMBA-treated animals (pre-neoplastic cells) contain the highest levels of DNA adducts of all groups, confirming a recent report.
that the DNA in these glands is extensively damaged (Wang et al. 2011). Second, at this
dose, E2 treatment does not modify the tumor growth rate, but it does increase the number
of DNA adducts, indicating that tumor cells are able to metabolize natural estrogens and
generate DNA adducts, increasing cellular instability. Third, MMI/T3 prevents the
increase of these E2-DNA adducts, showing that LPO has an important role in their
formation, and fourth, iodine treatment decreases the number of pre-established adducts
and prevents the formation of E2-generated adducts, suggesting the existence of specific
iodine-sensitive cells (cells with a high content of adducts?), and the competition between
iodide and estrogen for LPO (higher affinity of iodide versus E2 for LPO?). Together,
these data strongly suggest a complex interaction between E2 and iodine in mammary
carcinogenesis. In this context it is well established that moderately high concentrations
of estrogens (like pro-estrus levels) promote cancer progression, but that chronic, high
levels of E2 (characteristic of pregnancy) prevent both its initiation and progression
(Nandi et al. 1995; Sivaraman & Medina 2002). Many authors have proposed that these
effects are independent of nuclear receptors, suggesting catechol estrogens as an
important element (Lareef et al. 2005). This notion agrees with the report of Cavalieri’s
group showing that higher levels of E2-DNA adducts are present in the urine of breast
cancer patients and women at high risk for this disease (Cavalieri et al. 2010). Although
our study cannot exclude the participation of other enzymes (CYP1, CYP2, etc.) in DNA-
adduct generation, LPO is clearly implicated. Additional studies on the effects of iodine
in cells with high levels of DNA adducts and its influence on the production of catechol-
estrogens, both dependent on and/or independent of LPO are necessary; however, our
present data lead us to propose that iodine MIX (0.05% I2/0.05% KI) could be an
excellent component in breast cancer therapy, exerting apoptotic effects and inhibiting
the generation of DNA adducts without any side-effects on the thyroid or general
physiology.

**Declarations of interest**

The authors declare that they have no competing interests.

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**Authors' contributions**

OS and GD contributed equally to this work and carried out the cellular and molecular
studies, participated in the animal procedures, and drafted the manuscript. PP, EDMS,
and MEG carried out the DNA-adduct analysis and helped to draft the manuscript; BA
participated in the design of the study and performed the statistical analysis, and CA
participated in the study design and coordination. All authors read and approved the final
manuscript.

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Figure 1. Thyroid status in supplemented animals with different chemical and combinations of iodine and/or methimazole. Five-week-old rats were supplemented for 22 weeks with 0.05% or 0.07% molecular iodine (I₂), 0.1% potassium iodide (KI), 0.05%/0.05% I₂/KI (MIX), or 0.05%/0.1% I₂/KI (Lugol) with or without 0.05% methimazole + 3 µg/ml triiodothyronine (MMI/T₃) in the drinking water. Different superscripts indicate significant differences (p < 0.05).

Figure 2. Effect of moderately high concentrations of iodine/iodide and/or methimazole/T₃ on tumoral incidence. Five-week-old rats were treated for 22 weeks with 0.1% potassium iodide (KI), 0.05%/0.05% I₂/KI (MIX), or 0.05%/0.1% I₂/KI (Lugol) with or without 0.05% methimazole + 3 µg/ml triiodothyronine (MMI/T₃) in the drinking water. At 7 weeks of age, rats were treated with a single dose (i.g) of 20 mg DMBA. Data are expressed as the mean ± SD (n = 8). *p < 0.05, ** p < 0.01 compared to DMBA.

Figure 3. PPARγ induction and caspase-3 activity. Mammary glands and tumors of control or DMBA-injected animals treated or not with iodine MIX for 22 weeks were analyzed for PPARγmRNA expression by quantitative RT-PCR. Actin mRNA was amplified to check for RNA quantity and integrity. The experiments were repeated three times with independent RNA samples. Caspase-3 activity was analyzed by enzymatic assay (n = 5). Values are expressed as mean ± SD. Means with different letters are significantly different (p < 0.05).
Figure 4. Apoptosis rate. Formalin-fixed, paraffin-embedded sections of mammary glands and tumors treated or not with iodine MIX for 22 weeks were analyzed for apoptosis. A. Representative sections of TUNEL-positive cells revealed with diaminobenzidine (brown stain) and counterstained with hematoxylin (purple stain) (Magnification 20 X), B. TUNEL-positive cell quantification. Values are expressed as mean ± SD. Means with different letters are significantly different (p < 0.05).

Figure 5. LPO, PEN, and NIS expression. Mammary glands and tumors of control or DMBA-injected animals treated or not with iodine MIX for 22 weeks were analyzed for mRNA expression by quantitative RT-PCR. Actin mRNA was amplified to check for RNA quantity and integrity. Values are expressed as mean ± SD; the experiments were repeated three times with independent RNA samples. Means with different letters are significantly different (p < 0.05). One sample of lactating mammary gland was used as positive control.

Figure 6. Thyroid gland integrity. Thyroid glands from control and DMBA-treated animals after 22 weeks with or without iodine supplement. A, H&E-stained, 5-µm, paraffin-embedded sections (Magnification 40 X), B. Caspase-3 activity, analyzed by enzymatic assay. Means with different letters are significantly different (p < 0.05).

Figure 7. Effect of iodine, estrogen, and lactoperoxidase inhibition on number of DNA adducts and tumor growth. After 10 weeks of DMBA treatment, animals with
frank tumors were treated with 17β-estradiol (E₂; 0.5 µg/day), 0.05% I₂ + 0.05% KI (MIX), and/or 0.05% methimazole + 3 µg/ml T₃ (MMI/T₃) for 2 weeks. A. DNA-adduct content in control and DMBA-treated (damaged or frank tumors) mammary glands. Means with different letters are significantly different (p < 0.05). B. percent change in tumor growth. *p < 0.05 compared to control.
**PPAR-γ/actin**

- Ctrol
- DMBA
- MIX
- DMBA + MIX

**Caspase 3 activity (μMol pNA/mg protein/min)**

- Ctrol
- DMBA
- MIX
- DMBA + MIX

**Groups:**
- Mammary gland
- Tumor
A

DMBA Mammary gland

Control

MIX

Tumor

B

% TUNEL (positive cells /500 cells)

Mammary gland

Control

MIX

DMBA + MIX

Tumor

Ctrol DMBA MIX DMBA + MIX Ctrol MIX

Page 33 of 39
A

Control  |  DMBA  |  DMBA + 0.05% I$_2$

DMBA + MIX  |  DMBA + 0.1% KI  |  DMBA + Lugol

B

Caspase 3 activity

\[
\text{uMol pNA/mg protein/min}
\]

<table>
<thead>
<tr>
<th>Control</th>
<th>H$_2$O</th>
<th>0.05% I$_2$</th>
<th>0.1% KI</th>
<th>MIX</th>
<th>Lugol</th>
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</table>

DMBA
Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession</th>
<th>Sense / antisense sequence primer</th>
<th>Size (bp)</th>
<th>Alignment temperature (°C)</th>
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<tbody>
<tr>
<td>PPARγ</td>
<td>AF156665</td>
<td>TCAAACCCTTTACCACGGTT/CAGGCTCTACTTTGATCGCA</td>
<td>147</td>
<td>55</td>
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<tr>
<td>LPO</td>
<td>BF404752</td>
<td>CCTAGGAAGGGACAGTCGGATA/CCTCCTGTCTCCAGACTAGA</td>
<td>120</td>
<td>55</td>
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<tr>
<td>NIS</td>
<td>U60282</td>
<td>CCGGATCAACCTGATGGACT/CCTGAGGGTGCCACTGTAAG</td>
<td>377</td>
<td>60</td>
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<tr>
<td>PEN</td>
<td>AF167412</td>
<td>CATTCTGGGGCTGGACCTC/CCTCGGGCATCCTTCTTC</td>
<td>487</td>
<td>55</td>
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<tr>
<td>Cyc</td>
<td>M19533</td>
<td>AGACGCCGCTGTCTCTTTTC/GCCACACAGTCGGAGATGGTGATC</td>
<td>519</td>
<td>56</td>
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</table>

Peroxisome proliferator-activated receptor gamma, PPARγ; lactoperoxidase, LPO; sodium/iodide symporter, NIS; pendrin, PEN; cyclophilin, Cyc
Table 2. Effect of thyroid hormones, iodine, iodide, and/or methimazole on incidence, latency, and tumorogenesis rate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats with cancer</th>
<th>%</th>
<th>Cancer latency (week)</th>
<th>Tumorogenesis</th>
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<tr>
<td>Control</td>
<td>0/10</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>T4</td>
<td>0/10</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>0.1% KI</td>
<td>0/10</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>MIX</td>
<td>0/10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMBA</td>
<td>10/10</td>
<td>100</td>
<td>7</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>DMBA + T4</td>
<td>8/10</td>
<td>80</td>
<td>7</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>DMBA + T3</td>
<td>8/8</td>
<td>100</td>
<td>7</td>
<td>1.8 ± 0.7</td>
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<tr>
<td>DMBA + MMI/T3</td>
<td>7/8</td>
<td>87.5</td>
<td>8</td>
<td>1.5 ± 0.4</td>
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<tr>
<td>DMBA + 0.05 % KI</td>
<td>8/10</td>
<td>80</td>
<td>7</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>DMBA + 0.05 % I2</td>
<td>7/10</td>
<td>70</td>
<td>8</td>
<td>1.0 ± 0.3*</td>
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<tr>
<td>DMBA + 0.07 % I2</td>
<td>5/10</td>
<td>50</td>
<td>10*</td>
<td>1.0 ± 0.2*</td>
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</tbody>
</table>

* p < 0.05; significantly different from DMBA group
<table>
<thead>
<tr>
<th></th>
<th>Tumorogenesis</th>
<th>Tumor volume</th>
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<tbody>
<tr>
<td>DMBA</td>
<td>1.8 ± 0.7</td>
<td>2.4 ± 1.1</td>
</tr>
<tr>
<td>0.1% KI</td>
<td>1.3 ± 0.6</td>
<td>0.1 ± 0.1*</td>
</tr>
<tr>
<td>MIX</td>
<td>1.3 ± 0.6</td>
<td>0.07 ± 0.05*</td>
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<tr>
<td>Lugol</td>
<td>1.0 ± 0.0</td>
<td>0.02 ± 0.02*</td>
</tr>
<tr>
<td>MMI/T3</td>
<td>1.8 ± 1.0</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>MM/T3+ KI</td>
<td>1.2 ± 0.4</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td>MMI/T3 + MIX</td>
<td>1.5 ± 0.6</td>
<td>0.8 ± 0.5</td>
</tr>
</tbody>
</table>

* p < 0.05; significantly different from DMBA group