

# Uptake and antiproliferative effect of molecular iodine in the MCF-7 breast cancer cell line

*O Arroyo-Helguera, B Anguiano, G Delgado and C Aceves*

Instituto de Neurobiología, Universidad Nacional Autónoma de México, Campus Juriquilla Km 15 Carretera Qro-SLP, Juriquilla, 76230 Querétaro, México

(Requests for offprints should be addressed to C Aceves; Email: caracev@servidor.unam.mx)

## Abstract

This study analyzes the uptake and antiproliferative effect of two different chemical forms of iodine, iodide ( $I^-$ ) and molecular iodine ( $I_2$ ), in MCF-7 cells, which are inducible for the  $Na^+/I^-$  symporter (NIS) and positive for pendrin (PDS). The mouse fibroblast cell line NIH3T3 was used as control. Our results show that in MCF-7 cells,  $I^-$  uptake is sustained and dependent on NIS, whereas  $I_2$  uptake is transient with a maximal peak at 10 min and a final retention of 10% of total uptake. In contrast, no  $I^-$  was taken up by NIH3T3 cells, and although  $I_2$  was captured with the same time pattern as in MCF-7 cells, its uptake was significantly lower, and it was not retained within the cell. The uptake of  $I_2$  is independent of NIS, PDS,  $Na^+$ , and energy, but it is saturable and dependent on protein synthesis, suggesting a facilitated diffusion system. Radiiodine was incorporated into protein and lipid fractions only with  $I_2$  treatment. The administration of non-radiolabeled  $I_2$  and 6-iodo-5-hydroxy-8,11,14-eicosatrienoic acid (6-iodolactone, an iodinated arachidonic acid), but not KI, significantly inhibited proliferation of MCF-7 cells. Proliferation of NIH3T3 cells was not inhibited by 20  $\mu M$   $I_2$ . In conclusion, these results demonstrate that  $I_2$  uptake does not depend on NIS or PDS; they suggest that in mammary cancer cells,  $I_2$  is taken up by a facilitated diffusion system and then covalently bound to lipids or proteins that, in turn, inhibit proliferation.

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## Introduction

All vertebrates concentrate iodide ( $I^-$ ) in the thyroid gland for thyroid hormone (TH) synthesis (Carrasco 2000, Pisarev & Gartner 2000). The mechanism of  $I^-$  uptake involves active and passive transport systems present in several organs, including thyroid and mammary glands (Carrasco 2000, Soleimani *et al.* 2001, Rillema & Hill 2003a, Gillan *et al.* 2004). The active transport is mediated by  $Na^+/I^-$  symporter (NIS), which is a transmembrane glycoprotein that transports  $I^-$  against its concentration gradient and is perchlorate ( $KClO_4$ ) sensitive (Eskandari *et al.* 1997, Carrasco 2000). In lactating animals, NIS actively transports  $I^-$  from the maternal plasma to the alveolar epithelial cells of the mammary gland (Tazebay *et al.* 2000). In the human breast cancer cell line MCF-7, retinoic acid (RA) treatment increases NIS mRNA, NIS protein, and iodide uptake (Kogai *et al.* 2000).

Pendrin (PDS) is a facilitated diffusion transporter that is sensitive to disulfonic-2,2'-stilbene-4,4'-diisothiocianic acid (DIDS). PDS is also involved in  $I^-$  uptake and has been described in thyroid and mammary gland as well as in immortalized cell lines such as normal rat thyroid FRTL-5 (Royaux *et al.* 2000) and in several human breast cancer cell lines (Shennan 2001, Rillema & Hill 2003b, García-Solís *et al.* 2005a,b).

With regard to  $I_2$  uptake, studies in brown algae show that  $I^-$  uptake is dependent on oxidation, i.e. the  $I^-$  in seawater is oxidized to  $I_2$  or hypoiodous acid (HIO) by exohaloperoxidases and then penetrates into algal cells by means of a facilitated diffusion system (Küpper *et al.* 1998). Thyroid cancer cells transfected with the exoenzyme thyroperoxidase (TPO) or with both NIS and TPO (NIS/TPO) incorporate  $^{125}I^-$  into proteins, but cells transfected only with NIS do not. Moreover, in the presence of specific inhibitors of NIS or TPO, uptake and

protein organification of  $^{125}\text{I}^-$  is strictly dependent on TPO but not on NIS (Wenzel et al. 2003). These studies suggest that  $^{125}\text{I}^-$  is oxidized by TPO but does not use NIS to enter in the cell. Recent studies in our laboratory have shown that normal rat mammary glands and tumors take up  $\text{I}_2$  even in presence of  $\text{KClO}_4$  or furosamide, suggesting that  $\text{I}_2$  uptake does not depend on NIS or PDS respectively (García-Solís et al. 2005a,b). Several studies support the idea that the biological effect of  $\text{I}^-$  is mediated by iodinated derivatives, for example,  $\text{I}^-$  supplementation of cultured thyrocytes inhibits cell proliferation and induces apoptosis, effects shown only if TPO activity is present. Moreover, Vitale et al. (2000) showed that if TPO activity is blocked with 6-*n*-propyl-2-thiouracil (PTU), the apoptotic  $\text{I}^-$  effect is eliminated. In addition, in lung cancer cells transfected with NIS or NIS/TPO, the apoptotic effect is induced only in NIS/TPO transfected cells treated with  $\text{I}^-$  (Zhang et al. 2003). These data indicated that  $\text{I}^-$  must be oxidized in order to have a cytotoxic effect. In thyroid, this effect is mediated by iodinated arachidonic acid (AA) derivatives called: 6-iodo-5-hydroxy-8,11,14-eicosatrienoic acid or 6-iodolactone (6-IL) and/or by iodoheptadecanal (Dugrillon et al. 1990, Pisarev et al. 1994, Langer et al. 2003). In mammary gland, iodine deficiency is involved in dysplasias (Eskin et al. 1995, Aceves et al. 2005), which are reversible with  $\text{I}_2$  but not with  $\text{I}^-$  administration (Eskin et al. 1995). Recent data generated in our laboratory showed that continuous treatment with  $\text{I}_2$ , but not with  $\text{I}^-$ , has a potent antineoplastic effect on tumoral progression in *N*-methyl-*N*-nitrosourea-treated virgin rats (García-Solís et al. 2005a,b). The lack of  $\text{I}^-$  effect has been explained by the fact that lactoperoxidase, the enzyme that oxidizes  $\text{I}^-$  and covalently binds it to the milk protein, casein, is expressed in mammary gland only when this tissue is lactating (Strum 1978, Shah et al. 1986). In agreement with our findings, Shrivastava et al. (2006) reported that  $\text{I}_2$  treatment causes apoptosis in several human breast cancer cell lines but not in normal human peripheral blood lymphocytes. They showed the involvement of apoptosis-inducing factor (AIF) from mitochondria, which caused nuclear fragmentation independent of caspases. However, neither the  $\text{I}_2$ -uptake mechanism nor iodolipid formation has been investigated in mammary cells.

In this study, we used MCF-7 cells to test the participation of NIS and PDS in  $\text{I}_2$  uptake as well as its saturability and dependence on mechanisms, such as transcription, translation,  $\text{Na}^+$ , and energy. We also analyzed iodine incorporation into proteins or lipids (organification) and the effect of  $\text{I}_2$ ,  $\text{I}^-$ , and 6-IL on cellular proliferation.

## Materials and methods

### Materials and cell culture

Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-EDTA solutions were supplied by GIBCO-BRL (Grand Island, NY, USA). All *trans*-retinoic acid, DIDS, arachidonic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium azide, thimerosal,  $\alpha$ -amanitin, and silica gel were purchased from SIGMA Chemical Co. Cycloheximide (CHX) was obtained from Calbiochem (La Jolla, CA, USA). PTU was supplied by US Biochemical Co. (Cleveland, OH, USA). All other reagents were obtained from SIGMA Chemical Co. The breast cancer cell line MCF-7 (ATCC HTB-22) and mouse fibroblast cell line NIH3T3 (ATCC CRL-1658) were kindly supplied by Instituto Nacional de Cancerología de México and Instituto de Neurobiologías respectively. Cells were grown in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (basal medium) at 37 °C and 5%  $\text{CO}_2$  for 24 h before treatments.

### NIS induction and RT-PCR

To induce NIS, the cells were grown in six-well dishes in basal medium for 24 h, the medium was changed to HBSS and the cells were untreated or treated with 1  $\mu\text{M}$  RA for 48 h at 37 °C. After incubation, total RNA was extracted using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. To amplify 453 bp NIS, 625 bp PDS, and 545 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments, PCR primers with the sequences shown in Table 1 were used. GAPDH served as a control of RNA quantity and integrity, and the reverse transcriptase (RT)-PCR was carried out as described previously (Arroyo-Helguera et al. 2005). The samples were first denatured at 94 °C for 45 s, followed by 32 PCR cycles, each with temperature variations as follows: 94 °C for 45 s, 54.5 °C (GAPDH and NIS) or 60 °C (PDS) for 45 s, and 72 °C for 1 min. The last cycle was followed by an additional extension incubation of 3 min at 72 °C. The amplicons were resolved in a 2% agarose gel and visualized with ethidium bromide under a u.v. transilluminator. The sizes of the bands were confirmed by comparison with a commercial DNA ladder (1 kb DNA ladder, GIBCO-BRL).

Table 1 RT-PCR primer sequences

| mRNA                | GenBank accession no. | Sense/antisense primer sequence (5' at 3')           |
|---------------------|-----------------------|--|
| GAPDH, nt. 472–1017 | BC083511              | GACAACAGCCTCAAGATCATCAG/TTGCTGTAGCCAAATTCGTTGTAC     |
| PDS, nt. 1928–2553  | AF030880              | CACAGTTGGATTTGATGCCACTAGAGTA/TACGCATAGCCTCATCTGGACAT |
| NIS, nt. 630–1083   | NM-000453             | CTTCTGAACTCGGTCCTCAC/GAGCCGCTATACATTCTGGA            |

### Radioiodine-uptake assay

The procedure has been described (Kogai *et al.* 2000) but was used with minor modifications. Briefly, the cells were treated with or without RA and then incubated with 2.0 ml HBSS containing either 0.005  $\mu\text{Ci}$  of carrier-free  $\text{Na}^{125}\text{I}$  ( $^{125}\text{I}^-$ ; NEN Life Science Products, Boston, MA, USA) and 2  $\mu\text{M}$  KI or 0.005  $\mu\text{Ci}$  of  $^{125}\text{I}_2$  (obtained by the oxidation of  $^{125}\text{I}^-$  to  $^{125}\text{I}_2$  with  $\text{H}_2\text{O}_2$  and HCl as described by McAlpine (1945)) and 2  $\mu\text{M}$   $\text{I}_2$  (the final specific activity was 1.25 mCi/mmol in both cases). At the end of 2.5, 5, 10, 15, 30, and 60 min incubation, the plates were placed on ice, HBSS containing  $^{125}\text{I}^-$  or  $^{125}\text{I}_2$  was removed, and the cells were washed twice with 1 ml ice-cold PBS. The cells were scraped with 1 ml PBS, and the radioactivity was measured in a  $\gamma$ -counter. For uptake kinetics, the cells were incubated for 5 min with 0.005  $\mu\text{Ci}$   $^{125}\text{I}_2$  and 0.1–10  $\mu\text{M}$  unlabeled  $\text{I}_2$ .

### Effect of $\text{KClO}_4$ and DIDS on $\text{I}_2$ uptake

To analyze the participation of NIS or PDS in  $\text{I}_2$  uptake, cells with and without RA were preincubated for 15 min with  $\text{KClO}_4$  (5–50  $\mu\text{M}$ ) or DIDS (0.1–1 mM) respectively.  $^{125}\text{I}_2$  or  $^{125}\text{I}^-$  (1.25 mCi/mmol) was added to the medium, and it was kept at 37 °C for 15 min. After incubation, the cells were washed with PBS, and the radioactivity detected was normalized to  $10^4$  cells.

### Effect of transcription, translation, and metabolic inhibitors on $\text{I}_2$ uptake

To determine the effect of inhibiting RNA and protein synthesis on  $\text{I}_2$  uptake, the cells were preincubated for 30, 60, and 120 min in the presence or in the absence of 5  $\mu\text{g/ml}$  CHX or 5  $\mu\text{g/ml}$   $\alpha$ -amanitin, washed with PBS to remove the drugs, and subsequently incubated with 1.25 mCi/mmol  $^{125}\text{I}_2$  at 37 °C for 15 min. For metabolic inhibition, the cells were preincubated at 37 °C for 30 min in HBSS with and without 10 mM sodium azide or thimerosal. After washing twice, the cells were incubated with 1.25 mCi/mmol  $^{125}\text{I}_2$  or  $^{125}\text{I}^-$  at 37 °C for 15 min.

### $^{125}\text{I}_2$ and $^{125}\text{I}^-$ organification into proteins and lipids

The cells were treated with 100  $\mu\text{M}$  PTU, a TPO inhibitor, 5 min before adding 1.25 mCi/mmol  $^{125}\text{I}_2$  or  $^{125}\text{I}^-$  in HBSS medium; after 15 or 60 min, the cells were lysed with SDS-PAGE sample buffer containing the proteinase inhibitor cocktail Mini Complete (Roche) or precipitated with 10% trichloroacetic acid (TCA). The lipid fraction was extracted according to the procedure described by Bligh & Dyer (1959), and the radioactivity accumulated in the fractions was measured and expressed in c.p.m./ $10^6$  cells. The protein concentration in SDS-PAGE buffer lysates from cells treated with or without PTU was estimated using the Bradford assay (Bio-Rad); aliquots containing 50  $\mu\text{g}$  protein were heated at 70 °C for 3 min in 1 $\times$ SDS gel loading buffer (10% glycerol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8) with 2-mercaptoethanol) to reduce and denature protein and then separated by SDS-PAGE on 12% gels. Lipids were resolved by ascending thin-layer chromatography (TLC) using aluminium TLC sheets (silica gel 60 F254, layer thickness 0.2 mm, Merck) and dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), methanol (MeOH; 97.5:2.5% (v/v)) as solvent system. Lipids were visualized in an  $\text{I}_2$  vapor chamber and were identified by comparison with the standards of arachidonic acid (AA) and its 6-IL derivative run on the same plates. Radioiodinated proteins or lipids were exposed with a phosphor-intensifying screen and scanned in a Storm phosphoimager (Applied Biosystem). The masses of the protein bands were estimated by comparison with commercial molecular weight markers (high-range rainbow molecular weight markers 14.3–220 kDa, Amersham Biosciences).

### Synthesis of the 6-IL

The 6-IL was synthesized as described (Monteagudo *et al.* 1990). Briefly, 67 mg AA in 8.5 ml tetrahydrofuran and water (5 ml) was treated at 0 °C with potassium bicarbonate (50 mg), potassium iodide (133 mg), and  $\text{I}_2$  (203 mg) with continuous stirring for 4 days at 4 °C while protected from light. We then added saturated sodium thiosulfate solution. The radioactive iodolactone was synthesized by incubating 1 mCi  $^{125}\text{I}^-$  and 2.5  $\mu\text{l}$  chloramine-T (0.5 mg/ml

in 50 mM PBS) with 40 mg AA in 8 ml acetonitrile for 4 h at 0 °C. The product was extracted with CH<sub>2</sub>Cl<sub>2</sub>, washed with water, and dried with anhydrous sodium sulfate. The crude product was subjected to flash chromatography on silicagel (particle size, 230–400 mesh 60 Å). Elution was with CH<sub>2</sub>Cl<sub>2</sub>:MeOH (97.5:2.5%, v/v), fractions (5 ml) were collected and evaporated to dryness under nitrogen. Their purity was checked by TLC using CH<sub>2</sub>Cl<sub>2</sub>:MeOH (97.5:2.5%, v/v), and the spots were detected with I<sub>2</sub> vapor and autoradiography.

### Determination of cell proliferation

The effects of I<sup>-</sup>, I<sub>2</sub>, and 6-IL on cell proliferation were investigated using Trypan Blue dye exclusion and methylthiazolyldiphenyl-tetrazolium bromide (MTT) cell proliferation assays. The cells (3 × 10<sup>5</sup> cells/well) were cultured in DMEM supplemented with 1% FBS on six-well culture plates. The cells were then incubated with KI or I<sub>2</sub> (2–100 μM) and 6-IL (0.1–1 μM). Control cells were treated with water or with 0.1% ethanol, the solvents for the two iodine forms or 6-IL respectively. After 24, 48, and 72 h treatment, the cells were harvested with trypsin–EDTA. Trypan blue (0.4%) was added, and viable cells that excluded the dye were counted with a hemocytometer under the microscope. MTT was used for the cytotoxic evaluation. This assay is based on the conversion by viable cells of the yellow tetrazolium dye MTT to purple formazan crystals, and it provides an indirect, but quantitative determination of metabolically active cells. Briefly, the cells were seeded in a 96-well plate at a density of 5 × 10<sup>3</sup> cells/well and incubated for 24 h. After removing the medium, 200 μl fresh medium per well was added together with KI or I<sub>2</sub> (20 μM) and 6-IL (0.5 μM). After 24, 48, and 72 h, 10 μl MTT (5 mg/ml in PBS) were added to the wells, and the plate was incubated for 30 min at 37 °C in the dark. The medium was removed and 100 μl dimethyl sulfoxide was added to the wells, and the absorbance was measured at 550 nm using the SpectraCount ELISA reader. The percentage of proliferation was calculated by the equation

$$\% \text{ proliferation} = \frac{[(\text{test cell OD} - \text{control cell OD}) / \text{control cell OD}] \times 100.}$$

### Statistical analysis

The data were mean values of at least three different experiments and expressed as mean ± s.d. Differences between experimental groups were analyzed using a one-way ANOVA and Tukey's test and differences with *P* < 0.05 were considered statistically significant.

## Results

### Effect of RA treatment on NIS and PDS mRNA expression and radioiodine uptake

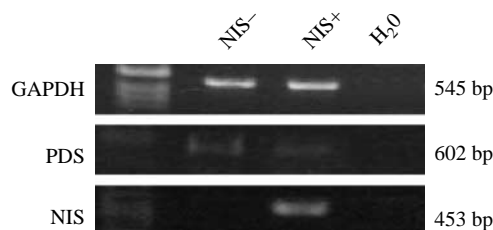
To evaluate the participation of NIS and PDS in I<sub>2</sub> uptake, the MCF-7 were treated with and without 1 μM RA for 48 h. Figure 1 shows that only untreated MCF-7 cells express PDS mRNA and that its expression does not change after RA treatment. In contrast, untreated MCF-7 does not express NIS mRNA, but its expression was induced by RA treatment. When radioiodine uptake was measured as shown in Fig. 2A, the MCF-7 cells without RA (NIS<sup>-</sup>) captured a minimal percentage of I<sup>-</sup> at 15 min. When MCF-7 cells were treated with RA (NIS<sup>+</sup>), <sup>125</sup>I<sup>-</sup> uptake increased gradually (Fig. 2A). For comparison, the cells with or without RA captured five times more I<sub>2</sub> than I<sup>-</sup> between 5 and 15 min. After 60 min of I<sub>2</sub> uptake, 10% of the radioactivity remained within the cells suggesting I<sub>2</sub> incorporation (Fig. 2B). As a non-mammary gland cell control, we used NIH3T3 cells, which neither express NIS mRNA nor capture I<sup>-</sup> (Tong et al. 1997). As expected, only basal I<sup>-</sup> uptake was observed (Fig. 2A). Although these cells capture a significant amount of I<sub>2</sub>, it is released completely by 60 min (Fig. 2B).

### Kinetics of I<sub>2</sub> uptake

The initial velocity of I<sub>2</sub> uptake was determined using 5-min incubation in 0.1–10 μM I<sub>2</sub> and 0.005 μCi <sup>125</sup>I<sub>2</sub>. The data show that the I<sub>2</sub> transport process was saturated above 5 μM I<sub>2</sub> (Fig. 3A). A Lineweaver–Burke plot yielded the apparent *K<sub>m</sub>* of 0.91 μM and a *V<sub>max</sub>* of 1.13 pmol/min per 10<sup>4</sup> cells (Fig. 3B), indicative of a high-affinity and high-capacity system.

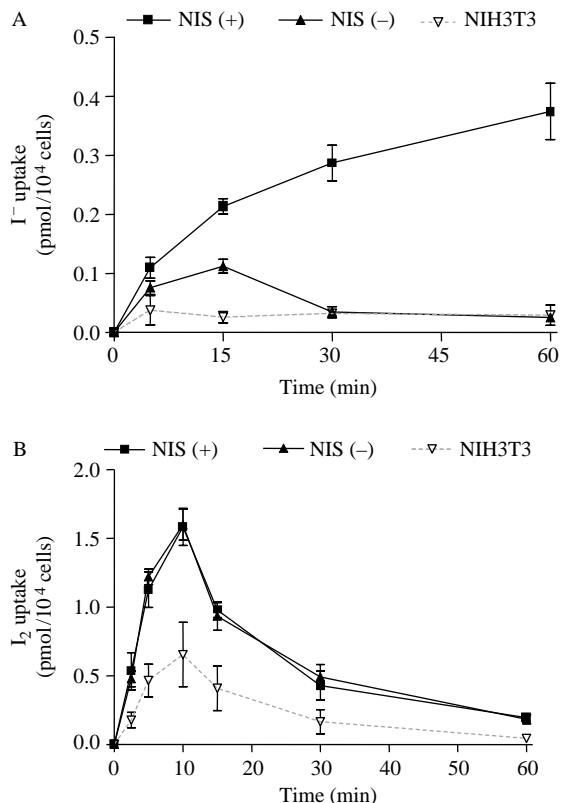
### Dependence of I<sub>2</sub> uptake on NIS and PDS transporters

MCF-7 cells with and without RA treatment were analyzed in the presence of different doses of DIDS or



**Figure 1** Expression of NIS and PDS mRNAs in MCF-7 cells. Representative electrophoresis (one of the three experiments with similar results). RT-PCR of total RNA from untreated cells (NIS<sup>-</sup>) or cells treated with 1 μM RA (NIS<sup>+</sup>) for 48 h. GAPDH mRNA was used as internal control for normalization purposes. H<sub>2</sub>O, water with all the PCR reagents except RT.



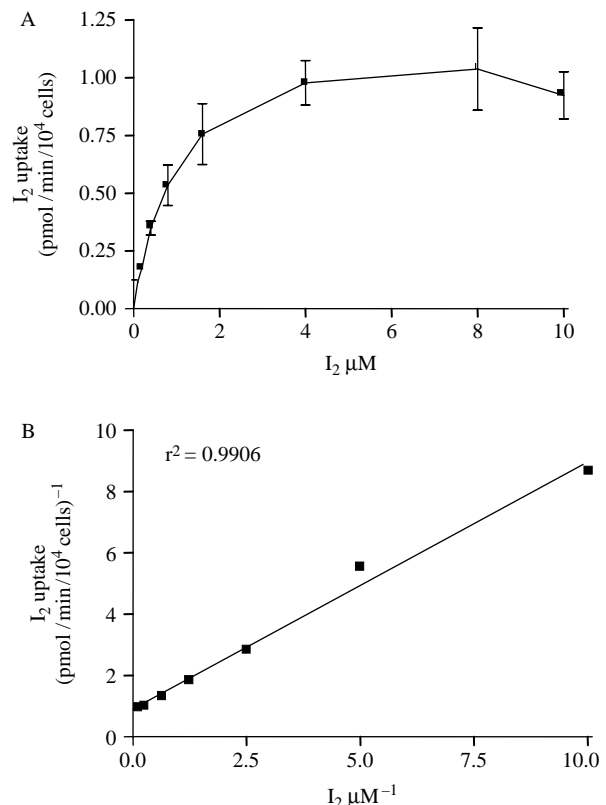


**Figure 2** Time-course of accumulation of different iodine forms in NIH3T3 and MCF-7 cells, with or without NIS expression. NIS (+) cells were incubated with  $1\ \mu\text{M}$  RA for 48 h to induce expression. All groups were treated with  $1.25\ \text{mCi}/\text{mmol}$   $^{125}\text{I}^-$  or  $^{125}\text{I}_2$  as indicated in Materials and methods. Radioiodine was determined with a  $\gamma$ -counter. A,  $^{125}\text{I}^-$  uptake. B,  $^{125}\text{I}_2$  uptake. The experiments were done in duplicate and repeated five times. Values are expressed as mean  $\pm$  s.d.

$\text{KClO}_4$ . Figure 4A shows that,  $1\ \text{mM}$  DIDS and  $30\ \mu\text{M}$   $\text{KClO}_4$  inhibited  $^{125}\text{I}^-$  uptake by over 50%, and with both drugs together the inhibition reached 70%. In contrast, they had no inhibitory effect on  $^{125}\text{I}_2$  uptake (Fig. 4B).

### Effect of transcription, translation, and metabolic inhibition on $\text{I}_2$ uptake

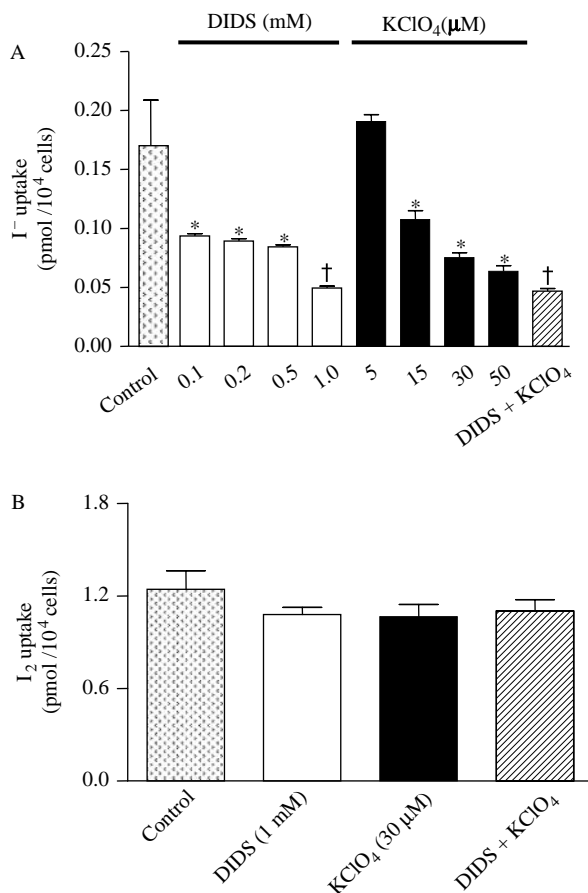
RNA and protein synthesis were inhibited by a preincubation with  $5\ \mu\text{g}/\text{ml}$   $\alpha$ -amanitin and CHX respectively.  $^{125}\text{I}_2$  uptake was not inhibited by  $\alpha$ -amanitin, whereas CHX caused 55% inhibition at 60 and 120 min (Fig. 5). The effect of metabolic inhibition on iodine uptake was measured with  $10\ \text{mM}$  sodium azide (electron transport inhibitor; Harvey *et al.* 1999) or  $10\ \mu\text{M}$  thimerosal ( $(\text{Na}^+/\text{K}^+)\text{-ATPase}$  inhibitor; Lewis & Bowler 1983). Figure 6 shows that  $^{125}\text{I}^-$  uptake was 40% inhibited with both treatments; in contrast, neither inhibitor affected the capture of  $^{125}\text{I}_2$ .



**Figure 3** Kinetics of  $\text{I}_2$  uptake. A, Michaelis-Menten kinetics expressed as mean  $\pm$  s.d. ( $n=3$ ). Cells were incubated with  $0.005\ \mu\text{Ci}$  of  $^{125}\text{I}_2$  in  $0.01\text{--}10\ \mu\text{M}$  unlabeled  $\text{I}_2$  at  $37\ ^\circ\text{C}$  for 5 min. B, Lineweaver-Burke double-reciprocal plot. The data are representative of three independent experiments. Each point is the mean of triplicate wells.

### Organization of $\text{I}_2$ or $\text{I}^-$

Figure 7 shows the radiolabeled  $\text{I}_2$  associated with protein (TCA precipitation) and lipid fractions at 15 and 60 min. The total radioactivity in the protein and lipid fractions represents 10–15 or 87–90% of the total radiolabeled  $\text{I}_2$  taken up by the cells at 15 or 60 min respectively. These incorporations were not affected by PTU treatment. In contrast, only a small quantity of the internalized  $\text{I}^-$  was incorporated into lipids, and no radioactivity was found in the protein fraction. Equal amounts of protein were separated on SDS-PAGE, and a representative experiment is shown in Fig. 8. Proteins of 18–20 kDa were labeled with  $^{125}\text{I}_2$ , but no proteins were labeled using  $^{125}\text{I}^-$ . Iodinated lipids were separated by TLC analysis in parallel with the standard samples of 6-IL (unlabeled), 6- $^{125}\text{I}$ IL (labeled), and AA and were visualized with  $\text{I}_2$  vapor or autoradiography. Representative results shown in Fig. 9 indicate that some of the iodinated lipid from  $^{125}\text{I}_2$ -treated cells comigrated with the 6-IL standard, whereas with  $^{125}\text{I}^-$  no discrete radioactive spots were

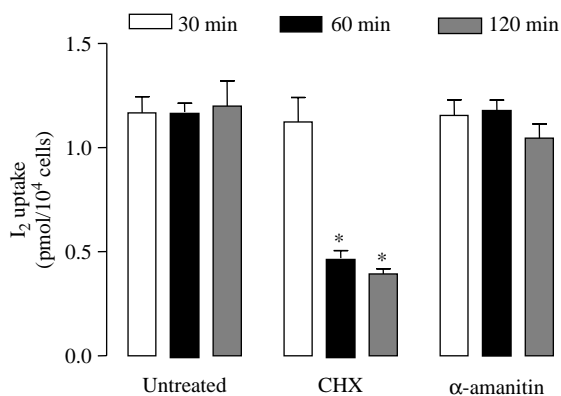


**Figure 4** Effect of PDS and NIS inhibitors on I<sup>-</sup> and I<sub>2</sub> uptake. After 48 h with (NIS +) or without 1 μM RA (NIS -), cells were incubated with the indicated concentrations of DIDS and KClO<sub>4</sub> for 30 min, then for 15 min with HBSS medium containing 1.25 mCi/mmol <sup>125</sup>I<sup>-</sup> or <sup>125</sup>I<sub>2</sub> and DIDS or KClO<sub>4</sub>. A, Effect of varying concentrations of DIDS and KClO<sub>4</sub> on I<sup>-</sup> uptake in NIS + MCF-7 cells. B, Effect of DIDS and KClO<sub>4</sub> on I<sub>2</sub> uptake in NIS - MCF-7 cells. Data are mean ± s.d. (n=5). \*P<0.05; †P<0.01, when compared with control cells.

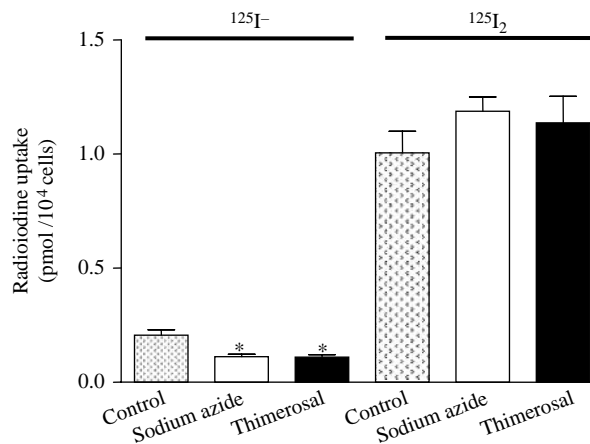
found. In addition, the incorporation of <sup>125</sup>I<sub>2</sub> was not significantly affected by 100 μM PTU (Figs 8 and 9).

### Cell proliferation

The effects of non-radioactive KI, I<sub>2</sub>, or 6-IL on MCF-7 cell proliferation were investigated using the Trypan Blue exclusion assay. As shown in Fig. 10, incubation with either I<sub>2</sub> or 6-IL for 72 h reduced cell proliferation in a dose-dependent manner. The inhibition was statistically significant with 20 and 100 μM I<sub>2</sub> and with 0.1, 0.5, and 1 μM 6-IL. Figure 11A shows the time-dependent effect of I<sub>2</sub> on proliferation of MCF-7 and NIH3T3 cells. After 48-h treatment with 20 μM I<sub>2</sub>, there was a significant reduction in MCF-7 cell numbers compared with untreated MCF-7 cells. The effect on proliferation of MCF-7 cells treated with 6-IL was significant beginning at 24 h (Fig. 11A). In contrast, NIH3T3 cell proliferation was not inhibited with 20 μM I<sub>2</sub> (Fig. 11A). Similar effects were observed using the MTT proliferation assay in MCF-7 and NIH3T3 cells treated with I<sub>2</sub> (Fig. 11B).



**Figure 5** Effect of inhibition of RNA and protein synthesis on I<sub>2</sub> uptake. Cells were preincubated for 5 min with 5 μg/ml α-amanitin or cycloheximide (CHX). Cells were then incubated with 1.25 mCi/mmol <sup>125</sup>I<sub>2</sub> at 37 °C for 15 min. Data are mean ± s.d. (n=3). \*P<0.05 versus control cells.



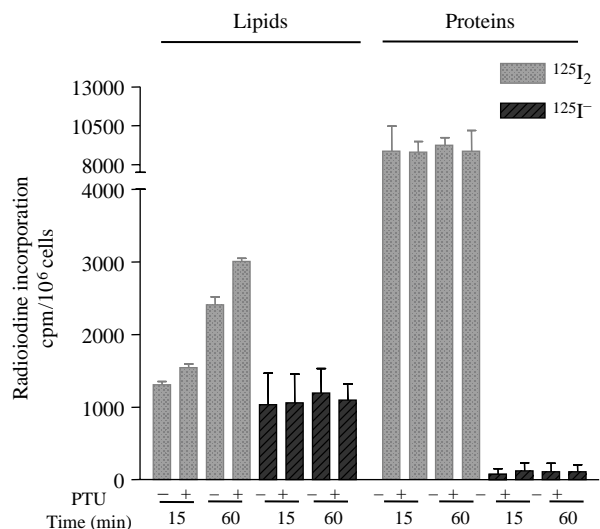
**Figure 6** Energy requirement of I<sub>2</sub> uptake in NIS+ MCF-7 cells. The cells were untreated or treated with 10 mM sodium azide or thimerosal for 30 min. After washing, they were incubated with 1.25 mCi/mmol <sup>125</sup>I<sub>2</sub> or <sup>125</sup>I<sup>-</sup> at 37 °C for 15 min. Data are mean ± s.d. (n=3). \*P<0.05 versus control cells.

## Discussion

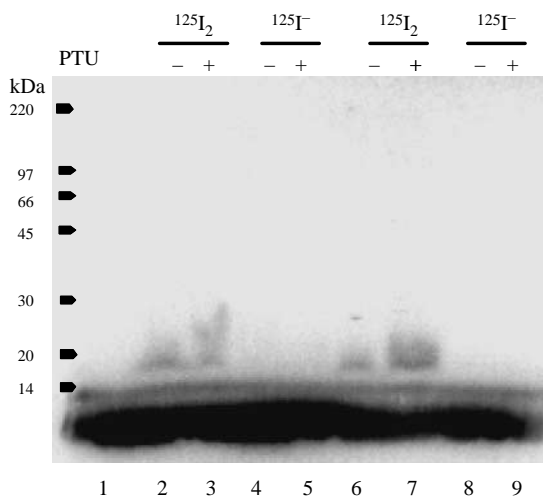
The present work shows for first time that the capture of I<sub>2</sub> occurs by a mechanism different from the transport of I<sup>-</sup>. Previous studies have demonstrated that NIS and PDS transporters are involved in I<sup>-</sup> uptake in both thyroid and mammary glands (Carrasco 2000, Rillema & Hill 2003a). In the present study, only RA-treated MCF-7 cells showed NIS mRNA expression and I<sup>-</sup> uptake, confirming that iodide internalization requires NIS (Kogai *et al.* 2000). Our findings show that in MCF-7 cells, with or without RA (NIS+ or NIS- respectively), I<sub>2</sub> uptake is five times greater than I<sup>-</sup> and has a peak within 5 and 15 min, suggesting that I<sub>2</sub> uptake does not require the NIS transporter. The NIH3T3 cells showed a similar pattern of I<sub>2</sub> uptake, but its capture was significantly lower. MCF-7 cells also express the PDS transporter, which is DIDS sensitive (Royaux *et al.* 2000, Rillema & Hill 2003b). Neither KClO<sub>4</sub> nor DIDS inhibited I<sub>2</sub> uptake in NIS+ MCF-7 cells, demonstrating that NIS and PDS do not participate in I<sub>2</sub> uptake. Our results suggest that I<sub>2</sub> uptake is mediated by a common facilitated transporter in mammary and non-mammary cells; however, in NIH3T3 cells the I<sub>2</sub> uptake is reduced, and I<sub>2</sub> not retained within the cell.

Our results show the existence of a distinct uptake system for I<sub>2</sub> that is saturable (> 5 μM) and has a high affinity (K<sub>m</sub> of 0.91 μM) and high velocity (V<sub>max</sub> of 1.13 pmol/min per 10<sup>4</sup> cells), contrasting with the low affinity and high velocity of I<sup>-</sup> uptake in NIS+ MCF-7 cells (K<sub>m</sub> of 21.9 μM and V<sub>max</sub> of 2.17 pmol/min per 10<sup>4</sup> cells; Kogai *et al.* 2000). In addition, I<sub>2</sub> uptake is dependent on protein synthesis, but it is independent of ATP and Na<sup>+</sup>/K<sup>+</sup>-ATPase. These characteristics were

reported by Rillema & Hill (2003b) for I<sup>-</sup> uptake by PDS, the DIDS-sensitive transporter. However, I<sub>2</sub> uptake by MCF-7 cells was not inhibited with DIDS. I<sub>2</sub> uptake followed Michaelis–Menten kinetics, but ATP and Na<sup>+</sup>/K<sup>+</sup>-ATPase were not required, suggesting a facilitated diffusion mechanism, according to the definition by Lobban *et al.* (1985). I<sub>2</sub> uptake showed similar characteristics in brown algae, where it was shown that after oxidation of I<sup>-</sup> to I<sub>2</sub> or HIO by haloperoxidases, oxidized iodine is captured by a facilitated diffusion system (Küpper *et al.* 1998).



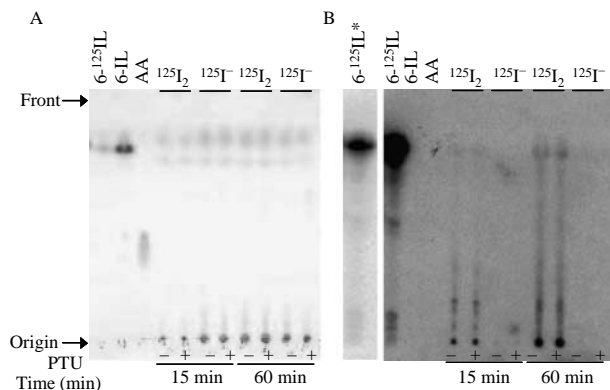
**Figure 7** Organization of I<sup>-</sup> versus I<sub>2</sub> into proteins and lipids. NIS- MCF-7 cells were incubated with or without 100 mM PTU for 5 min before adding 1.25 mCi/mmol <sup>125</sup>I<sub>2</sub> or <sup>125</sup>I<sup>-</sup>. The radioactivity of protein (TCA precipitate) and lipids (chloroform fraction) was expressed as c.p.m./10<sup>4</sup> cells. Data are mean ± s.d. (n=3).



**Figure 8** Analysis by SDS-PAGE of iodinated proteins from NIS – MCF-7 cells incubated with  $^{125}\text{I}^-$  or  $^{125}\text{I}_2$ . Cells were incubated with or without 100 mM PTU for 5 min before adding 1.25 mCi/mmol  $^{125}\text{I}_2$  or  $^{125}\text{I}^-$ . Aliquots containing 50  $\mu\text{g}$  protein were separated by 12% SDS-PAGE. Iodinated proteins were detected by autoradiography. 1, molecular weight markers; 2, 3, 4, and 5, radioiodinated proteins at 15 min; 6, 7, 8, and 9, radioiodinated proteins at 60 min. The figure is representative of electrophoresis of proteins labeled in five independent experiments.

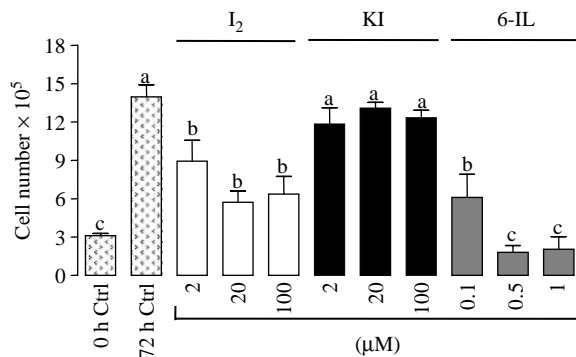
Several studies have reported that  $\text{I}^-$  needed to be oxidized by peroxidases, such as thyro-, myelo-, eosinophil-, and lactoperoxidases, and these, in turn, induced cytotoxic effects (Strum 1978, Boeynaems & Hubbard 1980, Turk et al. 1983, Ekholm & Bjorkman 1997). A specific species of oxidized iodine has not yet been identified, but several candidates exist, such as  $\text{I}^+$  (iodinium),  $\text{I}^0$  (iodine free radical),  $\text{IO}^-$  (hypoiodite),  $\text{HIO}$ , and  $\text{I}_2$  (Smyth 2003). Our results of organification in mammary cells show iodination of proteins with low-molecular weight. In lactating mammary gland,

iodine is incorporated into the milk protein, casein (Strum 1978, Shah et al. 1986), which is expressed by MCF-7 cells (Constantinou et al. 1998). It is possible that the lack of casein expression in mouse fibroblast cells explains why  $\text{I}_2$  is not retained within these cells. In mammary cells, we show that protein iodination took place in the presence of PTU, indicating that  $\text{I}_2$  organification does not require peroxidase activity. This result is consistent with previous studies showing that  $\text{I}_2$  generates thyroxine (T4) in the absence of peroxidase (Thrall et al. 1992). In contrast,  $\text{I}^-$  treatment did not generate iodinated proteins, which can be explained by the absence of peroxidase activity in MCF-7 cells (Kogai et al. 2000). Although we found radioactivity in the lipid fraction from  $\text{I}^-$  treated cells, when we analyzed it by TLC and autoradiography no iodinated spots were identified. Thus, the  $^{125}\text{I}^-$  we found in the lipid fraction might reflect a non-covalent interaction. It has been shown that  $\text{I}^-$  can bind to the lipid bilayer surface (Langner & Hui 1991). In contrast, lipids from cells treated with  $\text{I}_2$  showed a migration similar to the 6-IL standard, suggesting that  $\text{I}_2$  treatment could generate this type of iodolactone. In the thyroid gland, the antiproliferative and/or apoptotic effect of  $\text{I}^-$  treatment is mediated by iodinated arachidonic acid derivatives such as 6-IL or iodoheptadecanal (Dugrillon et al. 1990, Pisarev et al. 1994, Langer et al. 2003). *In vivo* iodolipid formation in mammary gland treated with  $\text{I}_2$  has not been investigated. Data generated in our laboratory showed a reduction in mammary tumors without changes in thyroid status (García-Solís et al. 2005a,b), suggesting a specific  $\text{I}_2$  effect only in tumoral tissue in rats. Several studies have reported elevated prostaglandin levels in breast cancer but not in normal mammary gland (Tan et al. 1974, Bennett et al. 1977, Rolland et al. 1980).



**Figure 9** Analysis of iodinated lipids from NIS – MCF-7 cells incubated with  $^{125}\text{I}^-$  versus  $^{125}\text{I}_2$ . Cells were incubated with or without 100 mM PTU 5 min before adding 1.25 mCi/mmol  $^{125}\text{I}_2$  or  $^{125}\text{I}^-$ . A, Representative TLC with iodinated lipids revealed by iodine vapors. B, Iodinated lipids on the same TLC detected by autoradiography after 15-h exposure. The experiment is representative of three different assays. 6- $^{125}\text{IL}$ , labeled 6-iodolactone; 6-IL\*, autoradiography of 6- $^{125}\text{IL}$  of the same TLC exposed only for 6 h.

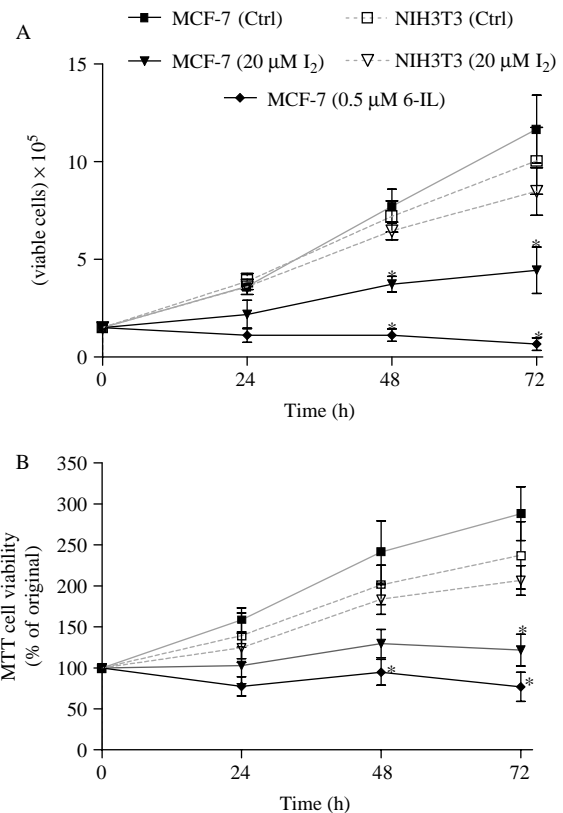




**Figure 10** Dose-dependent effects of  $I_2$ , KI, and the 6-IL on the proliferation of NIS- MCF-7 cells. Cells were incubated with different concentrations of KI,  $I_2$ , and 6-IL for 72 h. After the cells were stained with 0.4% Trypan Blue, viable cells were counted with a hemocytometer under the microscope. Data are expressed as mean  $\pm$  s.d. ( $n=5$ ). Different letters indicate significant differences between groups ( $P<0.05$ ).

Prostaglandins are produced from AA by the enzyme cyclooxygenase, indicating the presence of high levels of AA in breast tumors. It is possible that these high levels of AA, and the iodolipids formed from them, may explain the specific effect of  $I_2$  in tumoral cells. This hypothesis is being explored in our laboratory.

We also examined the effects of non-radioactive KI,  $I_2$ , or 6-IL, and we found that  $I_2$  and 6-IL treatments inhibited the proliferation rate of mammary cells in a dose-dependent manner at 72 h. When we analyzed the time dependence,  $I_2$  and 6-IL treatments inhibited proliferation within 24 h, but the mechanism of this inhibition has not been studied. In thyroid gland, iodine treatment arrested the cell cycle at the G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases (Tramontano *et al.* 1989, Smerdely *et al.* 1993). Other studies have shown an apoptotic effect induced by iodine excess in cultured thyrocytes. This effect did not involve changes in the antitumor protein p53 and did not require expression of apoptosis-related proteins, such as Bax, Bcl2, or Bcl XL (Vitale *et al.* 2000). With regard to the mammary gland, several authors have proposed that iodine deficiency alters the structure and function of human and rat mammary cells, and that  $I_2$  is more effective than  $I^-$  in diminishing ductal hyperplasia and perilobular fibrosis (Ghent *et al.* 1993, Eskin *et al.* 1995). Studies in our laboratory have shown that chronic administration of  $I_2$  but not  $I^-$  has a potent antineoplastic effect at the promotional level of mammary cancer and does not involve changes in the expression of p53 (García-Solís *et al.* 2005a,b). In addition to this, a recent report showed that the  $I_2$  effect in human breast cancer cell lines involves the activation and translocation of Bax from mitochondria, allowing the release and translocation of



**Figure 11** Proliferation curves of cells NIH3T3 and MCF-7 in the presence of  $I_2$  or 6-IL. After the indicated times aliquots were removed, and the cell number was determined by (A) direct counting using Trypan Blue exclusion, or (B) the MTT assay. Data are expressed as mean  $\pm$  s.d. ( $n=5$ ). \* $P<0.05$  versus control cells.

AIF to the nucleus, where it brings about nuclear fragmentation independent of caspases (Shrivastava *et al.* 2006). In the present study, we show that  $I_2$  treatment diminishes cell proliferation and is accompanied by iodination of lipids and proteins. We propose that the peroxisome proliferator-activated receptor (PPAR), a ligand-dependent transcription factor, can participate in the antiproliferative  $I_2$  effect. It is noteworthy that polyunsaturated fatty acids, including linoleic acid, eicosanoids, and AA (6-IL precursor), are endogenous PPAR ligands (Kliwer *et al.* 1997). PPAR ligands are involved in the regulation of cellular differentiation, cell cycle control, and apoptosis unrelated to p53 (Rosen & Spiegelman 2001, Shen & Brown 2003). Our results show that  $I_2$  treatment has an antiproliferative effect on MCF-7 cells, and they suggest the formation of 6-IL, which may mediate this effect; however, more detailed investigations are needed to elucidate the molecular mechanism of the actions of iodine derivatives in proliferation and/or apoptosis.

Taken together, these results demonstrate that I<sub>2</sub> uptake does not require NIS or PDS transporters and suggest that I<sub>2</sub> is taken up by a facilitated diffusion system and is subsequently bound to proteins or lipids that inhibit cell proliferation.

Finally, our findings both *in vivo* (García-Solís et al. 2005a) and *in vitro* (present work), as well the recent report of Shrivastava et al. (2006) showing that the antiproliferative effect of I<sub>2</sub> treatment is exerted only in tumoral cells, lead us to propose that I<sub>2</sub> treatment should be tested in clinical trials as an adjuvant of breast cancer therapy.

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