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# The effect of indole-3-carbinol on the expression of *CYP1A1*, *CYP1B1* and *AhR* genes and proliferation of MCF-7 cells

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**The influence of an antiestrogen, indole-3-carbinol (I3C) on the expression of *CYP1A1*, *CYP1B1* and *AhR* genes was investigated in an attempt to establish whether I3C could increase the expression of genes involved in estrone metabolism. Another purpose was to examine the proliferation of an estrogen-dependent breast cancer cell (MCF-7 line) under the influence of I3C and both I3C and DDT. In MCF-7 cells incubated with I3C or I3C and DDT combined, quantitative RT-PCR analysis revealed a significant increase in the level of *CYP1A1*, *AhR*, and *CYP1B1* transcripts. The proliferation rate of MCF-7 cells was increased by treatment with DDT or estradiol (E2), whereas I3C did not affect the proliferation of MCF-7 cells but greatly reduced the stimulatory effect of DDT, and abolished the effect of E2. The level of *p21* transcript, encoding p21 protein involved in the cell cycle, was increased several-fold by I3C comparing to its level in cells incubated with estradiol or DDT. The results suggest that the proliferation of MCF-7 cells is accompanied not only by expression of genes encoding cytochromes involved in estrogen metabolism, but also by changes in the expression of other genes including that encoding p21 protein involved in the cell cycle.**

**Keywords**: xenoestrogens, *CYP*, *AhR*, estrone hydroxylation, cell proliferation, p21

## INTRODUCTION

Cytochromes P450, the protein products of *CYP* genes are components of estrone hydroxylase enzyme system. Cytochromes P450 1A1 and P450 1A2 are responsible for 2-hydroxylation, which is the main route of estrogen elimination and provides a mechanism protecting the cells against cancer (Jefcoate *et al*., 2000). On the other hand, the action of cytochrome P450 1B1 involved in 4-hydroxylation results in the synthesis of potentially carcinogenic catechol estrogens. The expression of *CYP1A* and *CYP1B* gene families is mediated by aryl hydrocarbon receptor (AhR) (Safe, 2001).

Indole-3-carbinol (I3C) is a natural anti-estrogen isolated from *cruciferous* species (Michnovicz & Bradlow, 1990). Its affinity for estrogen receptor is weak and the anti-estrogenic properties are due to an increase of *CYP1A1* transcription and a decrease of the expression of proteins activating the cell cycle (Tiwari *et al*., 1994).

The main purpose of the present study was to examine the expression of *CYP1A* and *CYP1B1*, as well as *AhR*, in order to determine whether low doses of the natural anti-estrogen, I3C influence the expression of these genes in estrogen-dependent breast cancer cells (MCF-7 line). Studies on *CYP1A1* and *CYP1B1* expression have been reported (Brockdorff *et al*., 2000; Coumoul *et al*., 2001). However, the doses of xenoestrogens used in those experiments were very high and exceeded those found in the polluted environment. Another purpose of this study was to examine the effect of I3C, shown to protect cells against cancer (Bradlow *et al*., 1999), on estradiol (E2)-induced proliferation of MCF-7 cells, as well as the effect of this compound on cell proliferation in-

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**Abbreviations**: AhR, aryl hydrocarbon receptor; DDT, 1,1-bis-(4-chlorphenyl)-2,2,2-trichloroethane; E2, estradiol; ER, estrogen receptor; FCS, fetal calf serum; I3C, indole-3-carbinol.

duced by 1,1-bis-(4-chlorphenyl)-2,2,2-trichloroethane (DDT), considered as a breast cancer risk factor (Jaga & Brosius, 1999).

## MATERIALS AND METHODS

**Cell culture and incubation with test substances.** The estrogen-dependent MCF-7 cell line was kindly donated by Dr. S. Szala (Institute of Oncology, Gliwice, Poland). Cells were cultured at 37°C in 5% $CO_2$/95% air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotic-antimycotic (Sigma, UK) to reach approx. 80% confluency.

**Test substances.** I3C, DDT and estradiol (Sigma, UK) were dissolved in ethanol and kept at –20°C. Working solutions were diluted with the medium just before experiments to a final concentrations of: $10^{-8}$ M I3C, $10^{-6}$ M DDT and $10^{-12}$ M estradiol (E2). The final concentration of ethanol in the incubation medium did not exceed 0.01%.

**Expression assay.** MCF-7 cells were incubated with the test substances in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with ITS 100× Supplement Medium (1.0 mg/ml of insulin, 0.55 mg/ml of transferrin and 0.5 μg/ml of sodium selenite) (Sigma, UK). After 2 to 48 h of incubation the cells were counted and total RNA was isolated (Chomczynski, 1993) using TRI Reagent (Sigma, UK). Poly $A^+$ RNA was reverse-transcribed with the use of Superscript II RNase H Reverse Transcriptase (Invitrogen, USA) according to the producer's instructions, and cDNA fragments were amplified with the use of specific primers (Table 1). In order to quantify the concentration of specific mRNAs, real-time PCR was conducted using the LightCycler System (Roche Scientific, USA) and QuantiTect SybrGreen PCR kit (Qiagen, USA). The number of transcript copies was normalized to the number of cells (Bustin, 2002). Amplification of p21 cDNA was conducted in a total volume of 20 μl containing: 1 μl of template, 5 pmol of each primer, 200 μM of each dNTP, 2 μl of reaction buffer and 1 unit of RedTAQ Polymerase (Sigma, UK). Twenty-five PCR cycles, each consisting of: 5 s denaturation at 94°C, 20 s annealing at 64°C followed by 20 s elongation at 72°C. The amplified fragments were separated by electrophoresis in 2.5% agarose gel, analyzed by densitometry and quantified with the use of BandLeader software.

**Proliferation assay.** Cells were cultured for 48 h in DMEM without phenol red (Sigma, UK) supplemented with 10% fetal bovine serum devoid of steroids (Sonnenschein *et al*., 1995). When 30% confluence was reached, test substances were added to the final concentrations of: $10^{-8}$ M I3C, $10^{-6}$ M DDT and $10^{-12}$ M E2 and the cell proliferation monitored. At different time intervals, 5% MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] (Sigma, UK) was added. After 2 h of incubation the medium was removed and the cells were treated with DMSO (dimethylsulfoxide) (Sigma, UK) in order to visualize the metabolized MTT (Carmichael *et al*., 1987). Proliferation rate was expressed as the number of cells after 48 h of incubation with xenoestrogens compared to the untreated control.

**Statistics.** The results of expression and proliferation studies (each experiment was repeated three times) were subjected to ANOVA, while the results of *p21* expression were analyzed by Student's *t*-test. The significance of the differences was tested at the level of $P<0.05$.

## RESULTS

In MCF-7 cells incubated with I3C, the levels of *CYP1A1* and *CYP1B1* mRNA were significantly increased the latter being over 3-times higher than the former. The highest level of *CYP1A1* transcripts was detected after 4 h, while that of the *CYP1B1* transcripts after 2 h of treatment. A significant increase in *AhR* transcript appeared as early as 2 h of incubation with I3C and was maintained until 12 h. There was no change in the transcript levels upon incubation with estradiol, while DDT caused an expected increase in the expression of the three genes, with the highest levels being detected after 4 h (Fig. 1).

As expected, estradiol and DDT significantly enhanced proliferation of MCF-7 cells, I3C alone did not have any effect, but abolished the stimulatory effect of estradiol, and slightly, but significantly reduced the effect of DDT on cell proliferation (Fig. 2).

Semi-quantitative RT-PCR analysis revealed that the level of mRNA encoding an estrogen-dependent cell cycle protein, p21 was increased several-fold by I3C, while the effects of DDT and estradiol were negligible (Fig. 3).

## DISCUSSION

### The influence of I3C on the expression of *CYP1A1*, *CYP1B1* and *AhR*

An increased expression of *CYP1A1* under the influence of I3C in breast cancer cell lines and in mammary gland was described before (Horn *et al*., 2002), and an increase of CYP1A1 protein was observed after 12 h (Tiwari *et al*., 1994). Our results revealed the highest *CYP1A1* transcript level after 4 h of incubation with I3C, indicating that it pre-

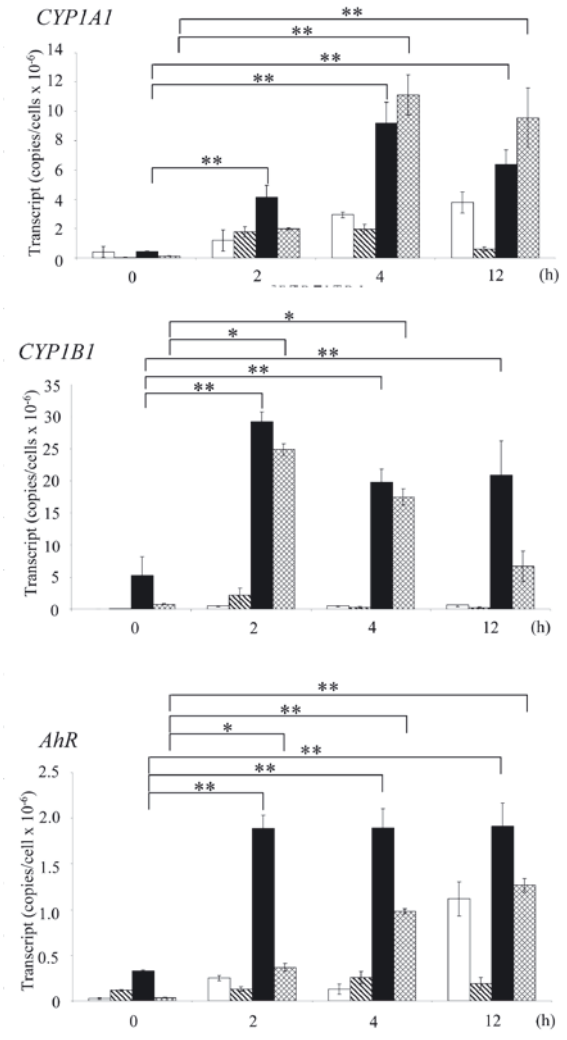


**Figure 1. Effects of I3C, DDT and estradiol on *CYP1A1*, *CYP1B1* and *AhR* expression.**
MCF-7 cells were incubated for the time indicated with $10^{-8}$ M I3C, $10^{-6}$ M DDT or $10^{-12}$ M estradiol in a defined medium (DMEM) devoid of phenol red. Following incubation, cells were counted, total RNA was extracted, poly $A^+$ RNA reverse transcribed and P450 1A1, P450 1B1 as well as AhR cDNAs were amplified and quantified by real-time PCR as described in Materials and Methods. Results were normalized to the cell number. Open bars, untreated control; hatched bars, E2; filled bars, I3C; double hatched bars, DDT. *$P$<0.05,**$P$<0.001 (Newman-Keul test).

ceded CYP1A1 protein accumulation. The increase in *CYP1A1* transcript level, in turn, was preceded by accumulation of *AhR* transcripts. This suggested that AhR mediated the effect of I3C on *CYP1A1* expression. Our results confirmed earlier observations that I3C and its metabolites in μM concentration induced the accumulation of AhR mRNA, followed by increased *CYP1A1* expression (Jellinck *et al*., 1993; Chen *et al*., 1998). However, the concentration of I3C used in those studies was five orders of magnitude higher than in the present report, and no kinetic data were presented. Therefore our observation on

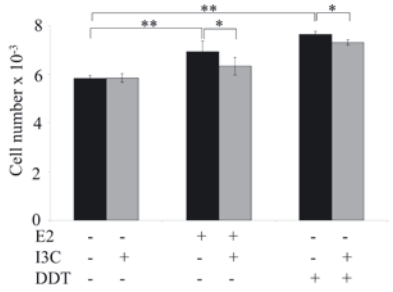


**Figure 2. Effect of I3C on estradiol- or DDT-induced proliferation of MCF-7 cells.**
MCF-7 cells in culture at 30% confluency were incubated for 48 h with I3C ($10^{-8}$ M), estradiol ($10^{-12}$ M) or DDT ($10^{-6}$ M) in DMEM containing 10% FBS, devoid of phenol red and steroids. Cell number was estimated by the MTT test as described in Materials and Methods. Results are the mean of three experiments, four dishes each. *$P$<0.05 (Newman-Keul test), **$P$<0.001 (Newman-Keul test).

a rapid accumulation of *AhR* transcripts under low doses (10 nM) of I3C should be considered as being novel and of physiological significance.

*CYP1B1* expression has been detected in both mammary gland and breast cancer cells (Hellmold *et al*., 1998; Iscan *et al*., 2001). *CYP1B1* expression was also demonstrated in human adrenocortical carcinoma cells treated with the I3C metabolite diindolemethane (Sanderson *et al*., 2001). However, in mammary gland of the rat (Horn *et al*., 2002), as well as in human prostate cells (Leibelt *et al*., 2003; Li *et al*., 2003), no I3C-induced *CYP1B1* expression could be demonstrated. On the other hand, in MCF-7 cells *CYP1B1* expression was induced by a typical agonist of AhR, TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), which exhibits a much higher affinity for AhR than I3C (Spink *et al*., 1998; Angus *et al*., 1999; Coumoul

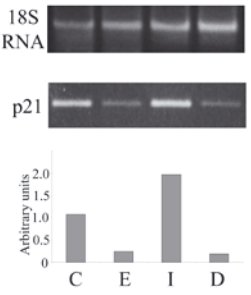


**Figure 3. Effect of xenoestrogens on *p21* mRNA content in MCF-7 cells.**
MCF-7 cells were grown to 50% confluency and were subsequently incubated for 24 h with I3C ($10^{-8}$ M), and/or DDT ($10^{-6}$ M) in DMEM containing 10% FBS, devoid of phenol red and steroids. After incubation total RNA was extracted and reverse transcribed. A fragment of *p21* cDNA (499 bp) was amplified by polymerase chain reaction (PCR) and the PCR product was analyzed by electrophoresis on 1.5% agarose gel. The experiment was repeated three times and typical gel is shown. (A) Ethidium bromide staining of agarose gel. (B) Densitometric analysis of the gel.

*et al.*, 2001). Our results demonstrate that in MCF-7 cells the *CYP1B1* expression induced by I3C was preceded by increased expression of *AhR*. Therefore it seems that I3C, thought to enhance the expression of the protective *CYP1A1*, also increases the expression of *CYP1B1*. In MCF-7 cells constitutive and induced expression of *CYP1B1* and only induced expression of *CYP1A1* was observed.

Steroid hydroxylases, related to cytochromes P450 1A1 and P450 1B1, catalyze 2-OH-E2 and 4-OH-E2 synthesis, respectively (Lee *et al.*, 2003). While stimulation of the formation of the 2-OH derivative formation protects the cells against potent estrogen accumulation, the 4-OH derivative accumulation is considered a breast cancer risk factor (Jefcoate *et al.*, 2000). Our results indicate that the protective effect of I3C is due to an enhanced *CYP1A1*/*CYP1B1* expression ratio rather than being caused by an increase in *CYP1A1* expression alone.

**The influence of indole-3-carbinol on proliferation of MCF-7 cells**

Our results were consistent with earlier reports (Liu *et al.*, 1994; Tiwari *et al.*, 1994; Diel *et al.*, 2002) and showed that both I3C and DDT affected the proliferation of the estrogen-dependent MCF-7 cells, but the inhibition by I3C of DDT-induced proliferation was never shown before. This effect of I3C might be due to changes in *CYP1A1*/*CYP1B1* expression ratio, and was AhR-mediated. DDT in concentrations used in our experiments was 100-times less effective in binding to the estrogen receptor (ER) than estradiol in a physiological concentration (Zava *et al.*, 1997). Consequently, I3C affects the metabolism of estrogens by changing the *CYP1A1*/*CYP1B1* expression ratio rather than by blocking the ER (Chen *et al.*, 1998).

Our results were supported by the examination of *p21* gene expression in MCF-7 cells. p21 ($p21^{CIP1}$) is a member of the CIP/KIP family of cyclin-dependent kinase inhibitors, suppressed by estrogens in a mechanism involving proteolysis and protein sequestration (Foster *et al.*, 2001). Similar to earlier studies, the concentration of *p21* gene transcript in MCF-7 cells under the influence of estradiol was low (Cariou *et al.*, 2000; Lai *et al.*, 2001). Sanders (1998) showed that I3C affected the expression of cell cycle proteins, but rather at the level of cyclin-dependent kinase 6 (CDK6) than kinase inhibitors. In our experiment I3C markedly enhanced the expression of *p21*, which was earlier demonstrated by Firestone and Bjeldanes (2003) and Cover *et al.* (1998) and indicated a protective role of I3C. The concentration of *p21* gene transcripts under the influence of DDT was as low as under the influence of estradiol, suggesting that the effects of xenoestrogens and natural estrogens on cell cycle protein expression in MCF-7 cells are similar.

The results of our study showed that I3C influences the expression of *CYP1A1* as well as *CYP1B1* and inhibits the proliferation of breast cancer MCF-7 cells induced by E2 and DDT. The protective affect of I3C might therefore be due not only to changes of the expression of *CYP* genes involved in estrogen metabolism, but also other factors including cell cycle proteins.

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