Biochemical Pharmacology, Vol. 58, pp. 1145–1153, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00197-5

Modulation of Cytochrome P4501A1 Activity by Ascorbigen in Murine Hepatoma Cells

Pernille Uldall Stephensen,†‡ Christine Bonnesen,*†‡ Leonard F. Bjeldanes† and Ole Vang*§*

*Department of Life Sciences and Chemistry, Roskilde University, DK-4000 Roskilde, Denmark; and †Division of Nutritional Sciences and Toxicology, University of California, Berkeley, CA 94720, U.S.A.

ABSTRACT. Modulation of cytochrome P4501A1 (CYP1A1) activity is a mechanism whereby indoles present in cruciferous vegetables could affect the metabolism of xenobiotics. Ascorbigen (ASG) is the predominant indole formed during the degradation of glucobrassicin, although the mechanism by which ASG modulates CYP1A1 activity is not known. The major focus of this study was to examine the mechanism of CYP induction by ASG using a murine hepatoma-derived cell line (Hepa 1c1c7). ASG was shown to induce the activity of 7-ethoxyresorufin O-deethylase, a marker for CYP1A1, in a concentration-responsive manner with a maximum induction at 700 μM. Maximum ASG induction after 24-hr treatment was 7% of maximal CYP1A1 activity induced by the well-known potent CYP1A1 inducer, indolo[3,2-*b*]carbazole (ICZ) (1 μM), and the EC_{50} values differed by 2-fold. The CYP1A1 activity increased continuously up to 72 hr, where ASG showed an induction efficiency in the same range as for the positive control (1 μM ICZ) after 24 hr, whereas the CYP1A1 protein level, measured by Western blot analysis, was maximally induced after 24 hr. ASG significantly inhibited CYP1A1 activity in whole cells at concentrations above 1 μM. ASG increased the chloramphenicol acetyl transferase (CAT) activity via a CAT reporter construct containing a dioxin-responsive element in Hepa 1c1c7 cells, indicating involvement of the aryl hydrocarbon receptor. ASG was shown to be transformed into ICZ, or a compound with the same chromatographic mobility as ICZ, in the medium. Taken together, the results indicate that ASG inhibits CYP1A1 activity at low concentrations, but induces the same activity at higher concentrations. BIOCHEM PHARMACOL **58**;7:1145–1153, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. ascorbigen; indolo[3,2-*b*]carbazole; Hepa 1c1c7 cells, *Cyp1a1* induction; *Cyp1A1* inhibition; Ah receptor

A number of studies have demonstrated a decreased risk of cancer when consuming a diet containing substantial amounts of fresh fruits and vegetables, especially cruciferous vegetables of the *Brassica* genus, e.g. broccoli, cabbage, and Brussels sprouts [1–3]. The anticarcinogenic activity of cruciferous vegetables has been related to the content of the glucosinolates; for example, glucobrassicin is present in large amounts in broccoli [4]. On disruption of plant tissue, such as cutting and chewing, different indoles are formed as a result of the myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1)-catalysed degradation of glucosinolates [5]. When glucobrassicin is degraded by myrosinase *in vitro*, I3C‖ is the main initial product formed at neutral pH. Under acidic conditions, condensation products of I3C, including DIM and ICZ, are formed (Fig. 1) [6, 7]. The yield of ICZ from the reactions of I3C under acidic conditions has been estimated to be 0.01% after 48 hr [8]. Glucobrassicin [9] and the degradation products formed have been shown to induce xenobiotic-metabolising enzymes, including the CYP1A1 enzyme, via the Ah receptor [8, 10]. By far the most potent Ah receptor agonist identified in the degradation mixture is ICZ [8].

Degradation of glucobrassicin in the presence of AA leads to the formation of ASG (Fig. 1) [11, 12]. AA is present in broccoli in amounts as high as 1.23 mg/g fresh broccoli [13], and it has been shown that ASG is the predominant indole compound in fresh, cooked, and fermented cabbage [7], about 7–10 times more abundant than I3C [7, 14]. Considering the abundance of ASG in vegetables of the *Brassica* genus, it seems that the biological effects of dietary ASG have been somewhat neglected. Thus far, only a few *in vivo* studies have investigated the effect of ASG on xenobiotic-metabolising enzymes [15]. McDanell *et al.* found that the EROD activity was increased by 45-fold in the small intestine and approximately 9-fold in the large intestine of rats fed a diet containing 1600 mg

‡ These authors contributed equally to the present study.

§ Corresponding author: Dr. Ole Vang, Department of Life Sciences and Chemistry, Roskilde University, DK-4000 Roskilde, Denmark. Tel. +45 4674 2552; FAX +45 4674 3011; E-mail: ov@virgil.ruc.dk

‖ *Abbreviations:* AA, ascorbic acid; Ah receptor, aryl hydrocarbon receptor; ASG, ascorbigen; CAT, chloramphenicol acetyl transferase; CYP, cytochrome P450; DIM, 3,3′-diindolylmethane; DRE, dioxin-responsive element; EROD, 7-ethoxyresorufin O-deethylase; ICZ, indolo[3,2-*b*]carbazole; I3C, indole-3-carbinol; RF, resorufin; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Received 25 August 1998; accepted 22 April 1999.

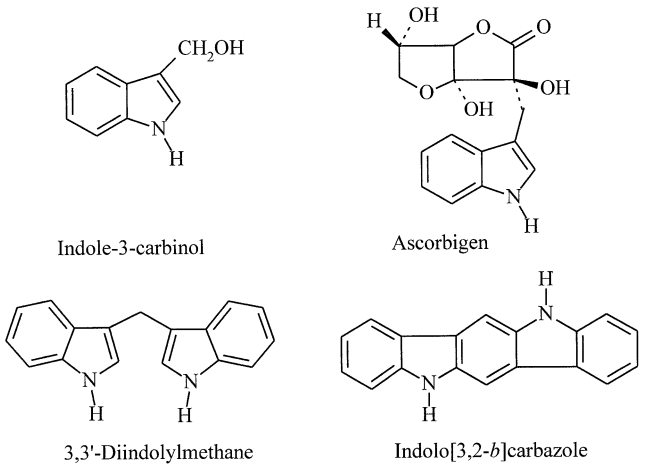

FIG. 1. Degradation products formed from glucobrassicin.

ASG/kg diet for 5 days, compared to controls [7]. Sepkovic *et al.* reported that ASG induces estradiol C-2 hydroxylation, primarily a CYP1A1 marker, about 1.5-fold in rats [16]. Inductions of CYP1A1-related activity have previously been observed for I3C and DIM in rats, and it has been suggested that this induction is responsible for altered carcinogen metabolism, leading to reduced tumour incidence [17]. The activity is most likely caused by oligomeric compounds formed from I3C and DIM, as I3C and DIM only weakly bind the Ah receptor and transcriptionally activate the *CYP1A* genes. It has also been shown that I3C and DIM both exhibit antagonist activity for the Ah receptor [18] and inhibit CYP activity. However, no results have been published on the mechanism by which ASG modulates CYP1A1 in cultured cells.

In the present study, we describe the CYP1A1-modulating activity of ASG using a murine hepatoma cell line (Hepa 1c1c7) and compare this activity with that of the potent CYP inducer, ICZ. Hepa 1c1c7 cells have been used in the present experiments as this cell line is stable, well characterised, and has been used for numerous experiments investigating Ah receptor-mediated induction of CYP1A1 [19].

MATERIALS AND METHODS

Chemicals

I3C was purchased from Aldrich Chemical Co. and recrystallised in toluene before use. ICZ and DIM were synthesised according to the procedure of Robinson [20] and Leete and Marion [21], respectively. TCDD was obtained from B. Ames (University of California, Berkeley, CA). 7-Ethoxyresorufin and RF were purchased from Sigma Chemical Co. and Aldrich Chemical Co., respectively. [RING,3,5-^{3}H] Chloramphenicol was purchased from Dupont New England Nuclear Co. *N*-Butyryl–coenzyme A lithium salt was from Sigma. All other organic solvents, chemicals, and biochemicals used in these studies were of the highest quality available from commercial sources.

General Methods

^{1}H-NMR spectra were measured at 300 MHz in methanol-d_4 on an AC-300 Brucker instrument. Chemical shift values were recorded relative to tetramethylsilane for all spectra. UV analysis was carried out on a Beckman UV/VIS spectrophotometer, model DU 530.

HPLC Analysis

For HPLC analysis, a C-18 bonded-phase column was used (Ultrasphere-ODS, 4.6 × 250 mm, particle size 5 μm, Beckman). The detector was either a Shimadzu UV-VIS spectrophotometer, model SPD-10A set at 280 nm, or a Perkin Elmer fluorescence spectrophotometer, model LS-4 set for excitation at 335 nm and emission at 415 nm. A gradient chromatographic and isocratic chromatographic system described by Aleksandrova *et al.* [14] and Kwon *et al.* [10], respectively, was used. The flow rate for both systems was 1 mL/min and the volume of injection 10 μL.

Synthesis of ASG

The synthesis was carried out according to the procedure of Kiss and Neukom [22], with some modifications. Briefly, 600 mg L-AA and 500 mg I3C were added to 125 mL H_2O and left stirred under nitrogen at room temperature for 2 hr. Light was avoided by wrapping the flask in foil. The solution was filtered and the aqueous phase neutralised with NaOH and extracted 3 times with ether. The aqueous phase was further extracted with ethylacetate and the extract was dried under reduced pressure (40°). Three hundred and fifty mg ASG was obtained as a white/pale yellow powder. The purity of ASG was assessed by HPLC and shown to be nearly 100%. AA, I3C, DIM, and ICZ were not detectable in preparations of ASG. The ^{1}H-NMR spectrum was identical to values reported previously [23]: ^{1}H-NMR (CHD_2-OH): δ 3.22 (1H, d), 3.40 (1H, d), 3.77 (1H, s), 3.98 (1H, dd), 4.10 (1H, dd), 4.20 (1H, dd), 6.97 (1H, t), 7.06 (1H, t), 7.20 (1H, s), 7.31 (1H, d), and 7.62 (1H, d). Additional parallel signals corresponding to about 10% isomeric ASG forms were observed in the NMR spectrum. The extinction coefficient for ASG was determined as 5650 $cm^{-1}M^{-1}$ at 280 nm.

Cell Culture Growth

The murine hepatoma cell line (Hepa 1c1c7 cells) and the Hepa cells stably transfected with the DRE reporter pMCAT 5.9 (M8 cells) were grown as monolayers at 37° in 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 3.7 g/L sodium bicarbonate, and 3.0 g/L glucose. pMCAT 5.9 is a plasmid constructed by ligation of the −820 to −974 enhancer sequence from the mouse promoter in the pMCAT 5 plasmid [24] and was provided by Dr. J. P. Whitlock, Jr. (Stanford University, Palo Alto, CA).

EROD Activity

Hepa 1c1c7 cells were grown in 58-mm diameter plates. ASG and ICZ, dissolved in either DMSO or PBS, were added to the cells ($9–14 * 10^4$ cells/cm^2) and incubated for various lengths of time. Control cell cultures were incubated with DMSO or PBS. As positive controls, cell cultures were incubated with 1 μM ICZ in DMSO. At the end of the incubation, cells were washed in PBS, harvested by trypsination, and resuspended in PBS. Two hundred μL cell suspension was used for cell counting. To 1.5 mL cell suspension, 0.5 mL 2.5 μM 7-ethoxyresorufin was added, and the EROD activity was determined by measurement of the linear production of the fluorescent RF at 37° on a Perkin Elmer 650-105 spectrofluorometer with 510 nm excitation, 585 nm emission, and a slit width of 20 nm. The amount of RF formed was determined by comparison with a standard curve (0–6.3 nM RF in PBS). The specific activity was calculated based on the actual number of cells.

Microsome Preparation and Inhibition of EROD Activity

Confluent Hepa 1c1c7 cells, treated for 24 hr with 1 μM ICZ, were washed twice in ice-cold PBS and harvested by use of a rubber policeman in PBS. The cells were sedimented and the pellet resuspended in phosphate buffer (50 mM $NaPO_4$, pH 7.4, 0.1 mM EDTA, 10% glycerol). The suspension was sonicated twice for 10 sec while kept on ice and the lysate centrifuged for 15 min at 10,000 *g* and 4°. The supernatant was further centrifuged for 1 hr at 100,000 *g* and 4° and the microsomal pellet resuspended in storage buffer (0.154 M KCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 20% glycerol). Microsomal protein concentration was determined by a Lowry assay using BSA as protein standard. The EROD inhibition assay was measured as an end point assay. Five hundred microliters of reaction mixtures consisting of 1.2 mg/mL BSA and 50 μg microsomes were prepared, dissolved in 0.1 M Tris–HCl buffer, pH 7.8, and incubated in a shaking waterbath at 37° with increasing concentrations of ASG (5–200 μM) for 10 min, following determination of the EROD activity. To the blank sample 1.25 mL MeOH was added, while 1 μM substrate solution (500 μM 7-ethoxyresorufin, dissolved in MeOH:H_2O, 1:1) and 0.5 mM NADPH were added to all samples, which were further incubated for 10 min. The reaction was stopped by addition of 1.25 mL ice-cold MeOH, and the samples were cooled at −20° for 30 min and centrifuged at 1800 *g* for 20 min. To one mL aliquots of the supernatants was added 10 μL 1 N NaOH and fluorescence was measured (Perkin-Elmer LS50B Luminescence Spectrometer) at excitation 530 and emission 585 nm. The standard curve was made using RF concentrations in the range 0–750 nM RF.

Western Blotting

The level of CYP1A1 protein in ASG-treated cells was determined using Western blotting and the enhanced chemiluminescence system (Amersham International plc). Following treatment, the cells were harvested in reporter lysis 5X buffer (Promega) and the lysates were resolved electrophoretically on a 12% constant SDS-PAGE gel using a Protean II system (Bio-Rad). The proteins were transferred to Hybond-P membrane (Amersham International plc) using a semi-dry blotting system [25]. Rabbit–anti-rat CYP1A1 from XenoTech LLC was used as primary antibody. The incubation and detection of the signals was performed as described by the manufacturer.

CAT Assay

Near confluent ($9–14 * 10^4$ cells/cm^2) M8 cells, Hepa 1c1c7 cells stably transfected with a CAT reporter gene, were treated for 19 hr with various concentrations of ASG (dissolved in DMSO) in the media ranging from 1 to 700 μM. The final concentration of DMSO in the media was 0.1% (v/v). Cells treated with DMSO or 1 μM ICZ were included as controls. CAT activity was measured in cell extracts by the two-phase extraction assay described by Seed and Sheen [26]. Briefly, cells were harvested and incubated for 5 min at room temperature in 5.0 mL buffer (20 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$). The buffer was aspirated and replaced by 100 μL of the buffer containing 0.1% Triton X-100. After 5-min incubation at room temperature, the lysate was centrifuged for 2 min to collect the supernatant. Assays were performed by incubating supernatants at 65° for 10 min. Fifty μL substrate was added to a final concentration of 100 mM Tris–HCl, pH 8.0, 100 μM [^{3}H]chloramphenicol (0.2 μCi), and 250 μM *N*-butyryl–coenzyme A. The mixture was incubated at 37° for 30 min and the reactions stopped by addition of 200 μL of 2,6,10,14-tetramethylpentadecane/xylene (2:1). After centrifugation, the organic phase was transferred to scintillation vials and counted.

Detection of ICZ Formed from ASG

Media from ASG kinetics experiments at 24 and 72 hr were extracted three times with ethylacetate and the organic phases evaporated to dryness. Samples were redissolved in 1 or 5 mL acetonitrile and analysed by HPLC. In parallel, the transformation of ASG in cell-free medium or PBS was estimated after various times of incubation at 37° or room temperature. Media were extracted and analysed as described above, whereas the PBS samples were analysed by HPLC without prior extraction. Authentic ICZ was used as a reference for determination of retention time and spiking of samples. To quantitate ICZ levels, authentic ICZ was added at various concentrations (10 nM–100 nM) to control cells preincubated for 24 or 72 hr before extraction.

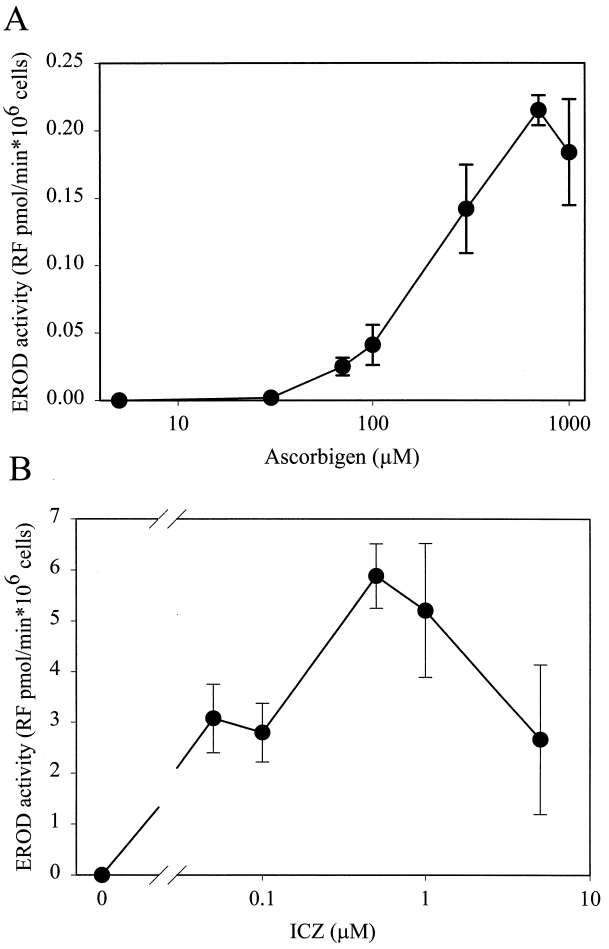

FIG. 2. Effect of ASG and ICZ on EROD activity in Hepa 1c1c7 cells. Cells were treated with increasing concentrations of ASG (1–1000 μM), panel A, or ICZ (0.05–5 μM), panel B, for 24 hr. The cells were then harvested for analysis of enzyme activity. Activity induced by solvent (DMSO) was subtracted for each concentration point. A positive control (1 μM ICZ) was included in the ASG experiment (panel A), i.e. 3.0 RF pmol/min/10^6 cells. Bars indicate mean values of three (panel A) or two (panel B) measurements ± SD (panel A) or range (panel B). The ASG induction experiment (panel A) was conducted twice with similar results.

RESULTS

Concentration-Dependent Induction of CYP1A1 Enzymatic Activity

The concentration–response effect of EROD activity induced by ASG and ICZ is shown in Fig. 2. The maximum induction response after 24 hr was achieved at 700 μM ASG, and the inducing efficiency of ASG was 7% of the EROD activity induced by the positive control, 1 μM ICZ. The EC_{50} values differed by nearly 2000-fold (Fig. 2, A and B). ASG is likely to modulate CYP1A1 activity in a manner similar to what has been reported for the other indoles derived from glucobrassicin. For example, ICZ binds to the Ah receptor and transcriptionally modulates CYP1A1 activity. To examine the effects of ASG on the Ah receptor, Hepa 1c1c7 cells transfected with a CAT reporter gene were treated with doses of ASG up to 700 μM

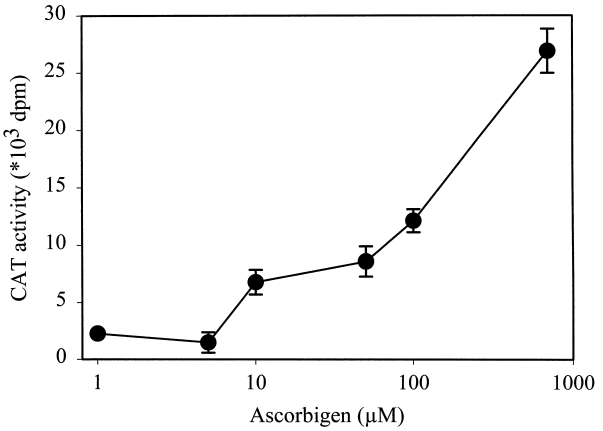

FIG. 3. Effect of ASG on DRE-driven CAT reporter gene activity in Hepa 1c1c7 cells. CAT reporter-transfected Hepa 1c1c7 cells (M8 cells) were treated for 19 hr with inducer at the indicated range of concentrations. Activity induced by solvent (DMSO) was subtracted for each concentration point. The CAT activity for the positive control (1 μM ICZ) was 67 * 10^3 dpm. Bars indicate mean values of two measurements ± range. The experiment was conducted twice with similar results.

(Fig. 3). Cells treated with 0.1% DMSO or 1 μM ICZ were included as controls. The CAT assay shows a concentration-responsive, ASG-induced CAT activity. Surprisingly, the activity of 700 μM ASG was approximately 40% of the CAT activity induced by 1 μM ICZ. A significant CAT activity was already observed at 10 μM ASG, i.e. approximately 25% of the highest observed activity at 700 μM ASG. Transcription of *Cyp1a1* requires a binding of the activated Ah receptor to DRE, which is located in the regulatory region of the *Cyp1a1* gene. Induction of CAT activity by ASG in the present experiment therefore indicates that the Ah receptor is involved in the ASG induction of *Cyp1a1*.

Concentration-Dependent Inhibition of EROD Activity

The greater efficacy exhibited by ASG for CAT induction compared to EROD induction, relative to ICZ, may arise from a strong inhibition of EROD activity by ASG. The inhibitory effect of ASG on microsomal EROD activity is shown in Fig. 4A. The result shows that ASG is an effective inhibitor of EROD activity above 50 μM in the ICZ-induced murine hepatoma microsomal system, as the activity declined with increasing ASG concentrations to 20% at the highest concentration of ASG analysed (200 μM). These results are in accordance with an experiment where Hepa 1c1c7 cells were co-treated with 1 nM TCDD, a highly potent inducer of EROD activity, and ASG at various concentrations for 24 hr, which gave a concentration-dependent decrease in EROD activity. The TCDD-induced EROD activity was significantly inhibited at concentrations above 1 μM ASG (Fig. 4B), thereby demonstrating the overall effect (induction and inhibition) of ASG on CYP1A1 activity. In conclusion, the results show

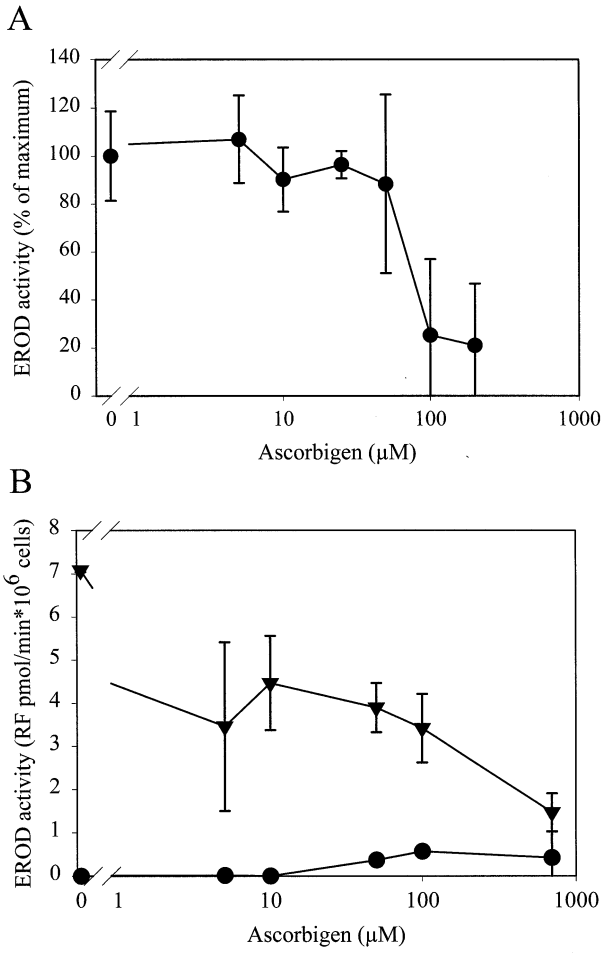

FIG. 4. Inhibitory effect of ASG on CYP1A1 enzymatic activity in microsomes and in whole cells. (A) Fifty μg ICZ-induced microsomes of Hepa 1c1c7 cells were treated with increasing concentrations of ASG 10 min before and throughout the determination of EROD activity. Bars indicate mean values of three measurements ± SD. The experiment was conducted twice with similar results. (B) Hepa 1c1c7 cells were treated with various concentrations of ASG alone (●) or co-treated with 1 nM TCDD and various concentrations of ASG (▼) for 24 hr followed by determination of EROD activity. EROD activity caused by DMSO was subtracted from each concentration point. Bars indicate mean values of two measurements ± range.

that beside activation of the Ah receptor, ASG also inhibits CYP1A1 activity.

Kinetics of Induction of EROD Activity by ASG

To investigate the kinetics of EROD induction by ASG, Hepa 1c1c7 cells were treated with 700 μM ASG for up to 84 hr, and the EROD activity was determined at different time points. The results (Fig. 5) indicate that the activity increases for up to 72-hr incubation with ASG dissolved in DMSO. The EROD activity after 72 hr was about 4-fold higher than that observed after 24-hr incubation. Since the solvent, DMSO, produces a weak CYP1A1 induction peak with a maximum at 8–12 hr (data not shown), we also

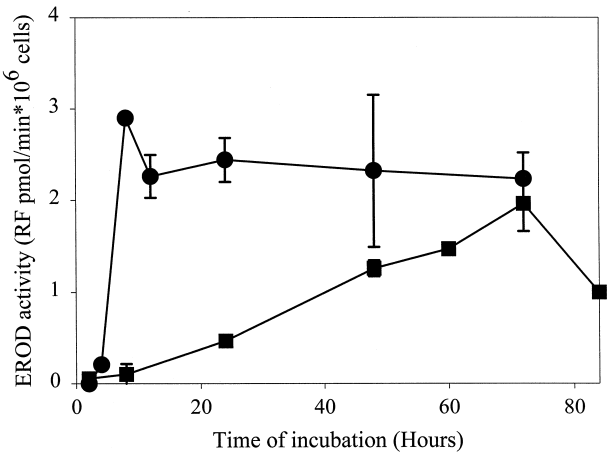

FIG. 5. Kinetics of EROD induction by ASG dissolved in DMSO or PBS in Hepa 1c1c7 cells. The cells were treated with 700 μM ASG dissolved in either DMSO (■) or PBS (●) and harvested at designated time points for analysis of enzyme activity. Activity induced by solvent (DMSO or PBS) was subtracted for each concentration point. Bars indicate mean values of two measurements ± range. The experiments were conducted twice with similar results.

analysed EROD activity kinetics in Hepa 1c1c7 cells exposed to ASG dissolved in PBS (Fig. 5). ASG (700 μM) induced EROD activity to a plateau (2.5 RF pmol/min/10^6 cells) after only 8 hr, which was the same activity obtained after 72 hr when using ASG dissolved in DMSO. This experiment shows that the kinetics for induction by ASG are different when DMSO and PBS are used as solvents.

Kinetics of Induction of CYP1A1 Protein Level by ASG

Hepa 1c1c7 cells were treated with 700 μM ASG, dissolved in DMSO, for up to 96 hr, and the CYP1A1 protein level was determined by Western blot analysis at different time points. The results (Fig. 6) show that the CYP1A1 level was maximally induced after 24 hr, with declining levels up to 96 hr. At 96 hr, the CYP1A1 protein level was reduced to 25% of the level at 24-hr ASG treatment, as determined by densitometric scanning. Comparison of the kinetics of EROD activity when ASG was dissolved in DMSO with the kinetics of the CYP1A1 protein level suggests that the time-dependent increase in EROD activity is caused by disappearance of the ASG from the medium, i.e. the inhibitory effect of ASG is reduced over time.

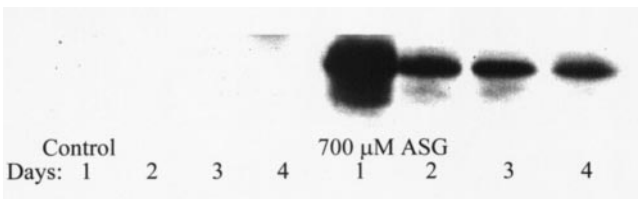

FIG. 6. Kinetics of CYP1A1 protein level induced by ASG. Hepa 1c1c7 cells were treated with 700 μM ASG, dissolved in DMSO, for 24, 48, 72, and 96 hr, and Western blot analysis was carried out on harvested cells using anti-CYP1A1 antibodies.

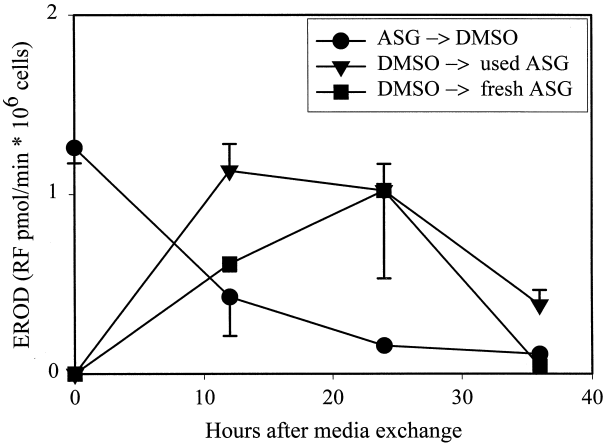

FIG. 7. Kinetics of EROD activity induced by degradation products of ASG. Cells were preincubated with 700 μM ASG (group 1, ●) or 0.1% DMSO (group 2, ▼, and group 3, ■) for 48 hr and EROD activity measured. At 48 hr, the media from group 1 and group 2 were swapped and further incubated for 36 hr. Group 3 was further incubated with fresh 700 μM ASG. EROD activity was measured at the designated time points. Bars indicate mean values of two measurements ± range.

EROD Induction by Degradation Products Formed from ASG during Incubation

To explain why a substance such as ASG, which only binds the Ah receptor weakly, may induce CYP1A1 activity, we suggest that ASG is transformed to an active inducer. Experiments to investigate this notion further were undertaken by determining the induction potential of preincubated ASG-containing medium (Fig. 7). Hepa 1c1c7 cells were preincubated with 700 μM ASG or DMSO for 48 hr followed by a media change as described below. EROD activity was analysed at different time points up to 36 hr after the media change. Cells were treated in three different ways. Cells in group 1 were exposed to ASG for 48 hr and then to an ASG-free medium. Two groups of cells were exposed to DMSO for 48 hr and then shifted to the 48-hr preincubated ASG-containing medium (group 2) or to a medium with fresh (700 μM) ASG (group 3). Figure 7 shows that EROD activity in cells preincubated with ASG decreased after exposure at 48 hr to the preincubated ASG-free medium (group 1). Twenty-four hours after replacement, the activity had decreased to 12% of that before replacement. In cells preincubated with solvent for 48 hr, the EROD activity 12 hr after the media exchange was virtually doubled when the 48-hr preincubated ASG medium (group 2) rather than the fresh ASG-containing medium was used (group 3). Cells exposed to preincubated ASG did show kinetics of EROD induction similar to those observed for CYP1A1 protein (Fig. 6). This experiment shows that preincubated ASG medium is a more effective inducer than fresh ASG, indicating transformation of ASG into a more active inducer. However, this experiment cannot exclude the possibility that the induction of EROD is in part caused by a simultaneous disappearance of ASG and thereby less inhibition of EROD activity.

HPLC Analysis of Degradation Products Formed from ASG

To test whether ASG was transformed into the potent EROD inducer, ICZ, in the cell media during incubation, 24- and 72-hr media from experiments where cells were exposed to ASG were analysed. The medium extracts were analysed by HPLC using fluorescence detection, since this method allows detection of many polycyclic aromatic compounds including ICZ. A peak with the same retention time as ICZ was observed and the identity of the peak was confirmed by spiking with authentic ICZ. The identity of this peak was not confirmed further. ICZ, or a substance with the same mobility, was detected in the medium after 24 hr, and the level was further increased about 5-fold at 72 hr. The level of ICZ or ICZ-like substance present in the ASG medium for 72 hr was estimated to be lower than 10 nM in comparison with controls (Fig. 8, A–C). In cell-free medium incubated with ASG for 2, 6, and 15 hr, ICZ was already detected after 2 hr (Fig. 8D) and found throughout the experiment.

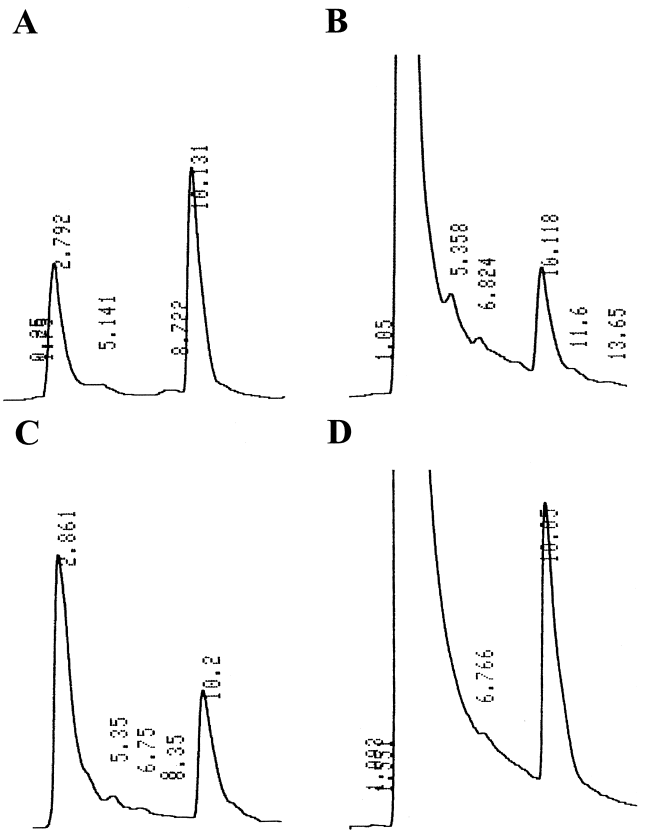

FIG. 8. HPLC analysis of extracts of media incubated with ASG. (A) Control medium to which ICZ (retention time 10.2 min) at the concentration 10 nM was added. (B) Medium from kinetic experiment at 24 hr with ASG dissolved in DMSO. (C) Medium from kinetic experiment at 72 hr with ASG dissolved in DMSO. (D) Cell-free medium incubated for 2 hr with ASG dissolved in DMSO. Following incubation, all media samples were extracted and redissolved in either 5 mL (samples A and C) or 1 mL (samples B and D) acetonitrile. HPLC conditions were as described in Materials and Methods.

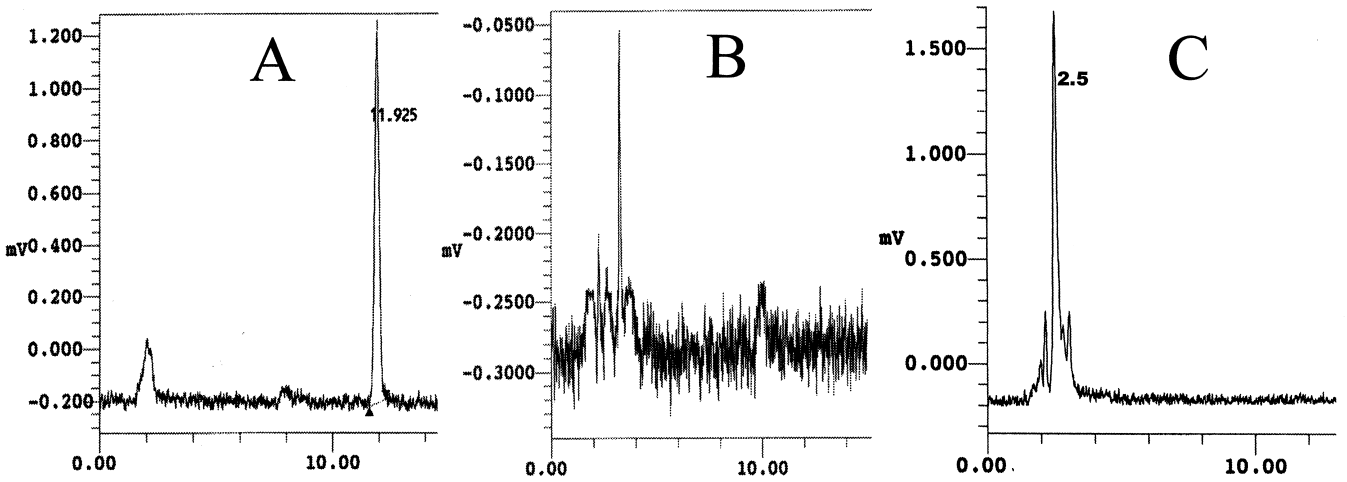

FIG. 9. HPLC analysis of PBS incubated with ASG. (A) Standard ICZ (5 nM). (B) 700 μM ASG incubated in PBS for 15 min at 37°. (C) 700 μM ASG incubated in PBS for 3 hr at 37°. HPLC conditions were as described in Materials and Methods.

In an attempt to account for the earlier EROD induction observed after exposure to ASG dissolved in PBS compared with DMSO, ASG dissolved in PBS was analysed by HPLC. No ICZ (retention time 11.9 min) was detected in the ASG–PBS solution either at room temperature or at 37° up to 24 hr. However, another fluorescent compound with a retention time of 2.5 min was found (Fig. 9). The fluorescent compound with a retention time of 2.5 min could also be formed in the ASG-containing medium, but detection of the compound was interfered with by the medium-specific signal with the same retention time. The fluorescent compound formed in PBS was not further characterised. In conclusion, ASG is transformed to ICZ (or a substance with an identical retention time) in the medium, but not in PBS buffer. However, the fluorescent compound found when ASG was incubated in PBS may be an EROD inducer or may be further transformed into ICZ.

DISCUSSION

It has been shown that broccoli contains high levels of the glucosinolate glucobrassicin and that this compound becomes degraded into I3C after the vegetable is processed. Under acidic conditions, as in the stomach, I3C will associate with the AA present in the vegetable to form ASG [27]. Numerous experiments have shown that I3C induces CYP1A1 activity following ingestion. As ASG is more prominent in cruciferous extracts, the inductive effect of ASG is of great interest. The purpose of this study was to investigate the mechanism of the *in vitro* modulation of CYP1A1 activity by ASG. ASG induced CYP1A1 activity in a concentration-dependent manner in the mouse hepatoma cell line, Hepa 1c1c7, with a maximum induction at 700 μM ASG. This concentration is about 700-fold higher than the maximum inducing concentration for ICZ found here (0.5–1 μM) and reported by Chen *et al.* [19]. The efficiency of ASG induction of CYP1A1 activity after 24 hr was 7% of the positive control (1 μM ICZ), whereas the level of ASG induced CAT activity corresponded to 40% of the ICZ-induced CAT activity. Moreover, the EC_{50} for induction of CAT activity was about one-half of the EC_{50} for induction of EROD, i.e. 120 μM and 210 μM, respectively. The promoter region of the CAT gene contains only one copy of mouse DRE whereas three DRE copies are found in the endogenous *Cyp1a1* promoter region, but these differences do not explain the observed differences in results obtained in the EROD and CAT assays. The likely explanation for these discrepancies could be that beside induction of EROD activity, ASG also exhibits an inhibitory effect on EROD activity. This assumption was confirmed in the present study, as ASG was shown to inhibit murine hepatoma microsomal EROD activity and TCDD-induced EROD activity in whole cells in a concentration-responsive manner. These profiles of responses Hepa 1c1c7 cells by ASG resemble those previously reported by Chen *et al.* [18] for I3C and DIM in T47D human breast cancer cells. These authors showed that both I3C and DIM are relatively weak Ah receptor agonists compared to ICZ and TCDD, since no induction of CYP1A1-dependent EROD activity was observed at concentrations as high as 125 and 31 μM, respectively [18], whereas I3C and DIM significantly inhibited TCDD-induced EROD activity in whole cells at concentrations of 31 and 1 μM, respectively. Furthermore, both I3C and DIM exhibited partial Ah receptor antagonist activity and significantly inhibited EROD activity in microsomes from TCDD-treated T47D cells at concentrations of 10 and 1 μM, respectively. In our experiments, the increase in EROD activity was shown to be caused by an induction of CYP1A1 protein by ASG, and the protein level was maximal after 24-hr exposure to ASG. Recently, Ciolino *et al.* [28] reported that another dietary constituent, curcumin, also has a dual property in being able to induce CYP1A1 in MCF-7 cells while also being an inhibitor of CYP1A1 activity.

The CAT experiment clearly indicates that ASG treatment activates the Ah receptor, which is a well-known

mechanism for induction of CYP1A1 protein and activity. Gillner *et al.* [29] previously demonstrated that the Ah receptor binding affinity of ASG, measured as IC_{50} values of the inhibition of specific [^{3}H]TCDD binding in rat liver cytosol, was more than 400 times lower than the corresponding value for ICZ and in the same range as for I3C. Since ASG has a very low affinity for the Ah receptor, it is likely that ASG is transformed into a more potent ligand and CYP1A1 inducer. ICZ, or a compound with an identical retention time, was detected in the ASG media at 24 and 72 hr. The possibility that other inducers are formed from ASG cannot be excluded. We therefore suggest that the induction of CYP1A1 protein by ASG is caused by transformation to the potent inducer ICZ and/or other potent products. Furthermore, it cannot be excluded from the present experiments that ASG may interact with other regulatory factors as well.

Preobrazhenskaya *et al.* [30] have previously shown that ASG incubated with gastric juice for 5 hr at 37° give rise to a very complex mixture containing ICZ, and that the concentration of ICZ in gastric juice was approximately 20 times higher after incubation under these conditions with ASG than after incubation with I3C. Our experiments indicate that ICZ may also be formed from ASG under physiological conditions at neutral pH. In contrast, ASG dissolved in PBS caused an EROD induction with the same efficiency at 8 hr as found for the positive control at 24 hr. This induction pattern corresponds to the ICZ-induced EROD activity shown by Chen *et al.* [19]. One can therefore speculate that ASG is transformed to ICZ in the aqueous buffer before addition to the medium. However, ICZ was not detected using HPLC in the ASG–PBS solution when incubated at 37° for up to 24 hr, whereas another fluorescent, more polar compound was detected. The identity of this compound has not been established but the ASG oligomers do not exhibit fluorescence, and the unidentified compound is likely to be a polycyclic polar product. The early response in EROD activity observed when using PBS as ASG solvent is therefore not caused by the presence of ICZ in the initial PBS solution. Furthermore, the low level of ICZ detected in the media cannot by itself account for the entire CYP1A1 induction observed. Degradation of ASG is likely to result in the release of AA, and the effect of AA on induction of CYP enzymes is not well characterised. It cannot be excluded that AA *in vitro* may enhance the inducing potential of ASG/ICZ. The observed EROD kinetic is a combination of a fast induction by ICZ or an ICZ-like compound, ASG, and other potential inducers formed from ASG and a slow disappearance of the inhibitor ASG from the medium over time. The half-life of ICZ is about 10 min [19], and the effect presented here is likely caused by a continuous formation of ICZ from an excess amount of ASG. Induction of CAT is observed above 10 μM ASG, but because of a significant ASG-mediated inhibition in whole cells above 1 μM, a significant induction of EROD is only observed above 50 μM ASG.

In conclusion, our results show that exposure to ASG induces CYP1A1 expression in Hepa 1c1c7 cells. ASG treatment induces Ah receptor-driven CAT activity and therefore presumably induces CYP1A1 via activation of the Ah receptor. According to the literature, ASG acts as a weak Ah receptor agonist compared to ICZ or an ICZ-like substance, but the induction may in part be explained by the transformation of ASG to the more potent inducer ICZ. In addition to inducing EROD activity, ASG also inhibits this activity. As ASG is the main transformation product from glucobrassicin in the presence of AA, the observation that ICZ, or an ICZ-like substance, may also be formed at neutral pH is of great interest.

We thank Charlene Schaldach (University of California, Berkeley, CA) for undertaking the CAT assay, Dr. Simon Bolvig (University of California, Berkeley, CA) for the NMR analysis, and Dr. Niels Agerbirk (The Royal Veterinary and Agricultural University, Denmark) for helpful discussions on ASG synthesis. Valuable comments on the manuscript from Dr. Hanne-Cathrine Bisgaard (Roskilde University) are appreciated. This work was supported by the Danish Cancer Society (P. U. S.), the Wedell-Wedellsborg Foundation (P. U. S., C. B., O. V.), The Foundation for Disease Treatment without use of Animal Experiments (O. V.), the US Department of Defense, Army Breast Cancer Research Program Grant DAMD17-96-1-6149 (L. F. B.) and NIH Grant CA 69056 (L. F. B.).

References

1. Steinmetz KA and Potter JD, Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Causes Control* **2:** 325–357, 1991.
2. McDanell R, McLean AEM, Hanley AB, Heanley RK and Fenwick GR, Chemical and biological properties of indole glucosinolates (glucobrassicins): A review. *Food Chem Toxicol* **26:** 59–70, 1988.
3. Prochaska H, Santamaria AB and Talalay P, Rapid detection of inducers of enzymes that protect against carcinogens. *Proc Natl Acad Sci USA* **89:** 2394–2398, 1992.
4. Bjerg B and Sørensen H, Isolation of intact glucosinolates by column chromatography and determination of their purity. In: *Glucosinolates in Rapeseeds: Analytical Aspects*. (Ed. Wathelet J), pp. 59–75. Martinus Nyhoff, Dordrecht/Boston/Lancaster, 1987.
5. Sørensen H, Glucosinolates: Structure-Properties-Function. In: *Rapeseed/Canola: Production, Chemistry, Nutrition and Processing Technology* (Ed. Shahidi F), pp. 149–172. Van Nostrand Reinhold, New York, 1990.
6. Bradfield CA and Bjeldanes LF, Dietary modifications of xenobiotic metabolism: Contribution of indolylic compounds present in *Brassica oleracae*. *J Agr Food Chem* **35:** 896–900, 1987.
7. McDanell R, McLean AEM, Hanley AB, Heaney RK and Fenwick GR, Differential induction of mixed-function oxidase (MFO) activity in rat liver and intestine by diets containing processed cabbage: Correlation with cabbage levels of glucosinolates and glucosinolate hydrolysis products. *Food Chem Toxicol* **25:** 363–368, 1987.
8. Bjeldanes LF, Kim J, Grose KR, Bartholomew JC and Bradfield CA, Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*: Comparison with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Proc Natl Acad Sci USA* **88:** 9543–9547, 1991.
9. Bonnesen C, Stephensen PU, Andersen O, Sørensen H and

Vang O, Modulation of cytochrome P-450 and glutathione *S*-transferase isoform expression *in vivo* by intact and degraded indolyl glucosinolates. *Nutr Cancer* **33:** 178–187, 1999.
10. Kwon C, Grose KR, Riby J, Chen Y and Bjeldanes LF, *In vivo* production and enzyme-inducing activity of indolo[3,2-*b*]carbazole. *J Agr Food Chem* **42:** 2536–2540, 1994.
11. Kutacek M, Prochazka Z and Veres K, Biogenesis of glucobrassicin, the *in vitro* precursor of ascorbigen. *Nature* **194:** 393–394, 1962.
12. Preobrazhenskaya MN, Korolev AM, Plikhtyak IL, Yartseva IV, Efimov SA, Lazhko EI and Alexandrova LG, Chemistry and biology of acsorbigens. In: *Heterocycles in Bio-organic Chemistry* (Eds. Bergman J, Van der Plas HC and Simonyi M), pp. 68–86. The Royal Society of Chemistry, Cambridge, 1991.
13. Moller A, *Levnedsmiddeltabeller* 4. revised ed. (Ed. Saxholt E). National Food Agency, ISBN 87-601-4742-3, 1996.
14. Aleksandrova LG, Korolev AM and Preobrazhenskaya MN, Study of natural ascorbigen and related compounds by HPLC. *Food Chem* **45:** 61–69, 1992.
15. Vang O and Dragsted L, *Naturally Occurring Antitumourigens—III. Indoles*. The Nordic Council of Ministers, Copenhagen, 1996.
16. Sepkovic DW, Bradlow HL, Michnovicz J, Murtezani S, Levy I and Osborne MP, Catechol estrogen production in rat microsomes after treatment with indole-3-carbinol, ascorbigen, or β-naphthaflavone: A comparison of stable isotope dilution gas chromatography-mass spectrometry and radiometric methods. *Steroids* **59:** 318–323, 1994.
17. Wattenberg LW, Inhibition of neoplasia by minor dietary constituents. *Cancer Res* **43:** 2448–2453, 1983.
18. Chen I, Safe S and Bjeldanes LF, Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. *Biochem Pharmacol* **51:** 1069–1076, 1996.
19. Chen Y, Riby J, Srivastava P, Bartholomew J, Denison M and Bjeldanes LF, Regulation of CYP1A1 by indolo[3,2-*b*]carbazole in murine hepatoma cells. *J Biol Chem* **270:** 22548–22555, 1995.
20. Robinson B, The Fisher indolisation of cyclohexane-1,4-dione bisphenylhydrazone. *J Soc Chem* **:** 3097–3099, 1963.
21. Leete E and Marion L, The hydrogenolysis of 3-hydroxymethylindole and other indole derivates with lithium aluminium hydride. *Can J Chem* **31:** 775–784, 1953.
22. Kiss G and Neukom H, Uber die struktur des ascorbigens. *Helv Chim Acta* **49:** 989–992, 1996.
23. Agerbirk N, Olsen CE and Sørensen H, Initial and final products, nitriles and ascorbigens produced in myrosinase catalysed hydrolysis of indole glucosinolates. *J Agr Food Chem* **46:** 1563–1571, 1998.
24. Fisher JM, Wu L, Denison MS and Whitlock JP Jr, Organization and function of a dioxin-responsive enhancer. *J Biol Chem* **265:** 9676–9681, 1990.
25. Vang O, Jensen H and Autrup H, Induction of cytochrome P-450IA1, IA2, IIB1, IIB2 and IIE1 by broccoli in rat liver and colon. *Chem Biol Interact* **78:** 85–96, 1991.
26. Seed B and Sheen J, A simple phase-extraction assay for chloramphenicol acyltransferase activity. *Gene* **67:** 271–277, 1988.
27. Piironen E and Virtanen AI, The synthesis of ascorbigen from ascorbic acid and 3-hydroxymethylindole. *Acta Chem Scand* **5:** 1286–1287, 1962.
28. Ciolino HP, Daschner PJ, Wang TT and Yeh GC, Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. *Biochem Pharmacol* **56:** 197–206, 1998.
29. Gillner M, Bergman J, Cambillau C, Fernström B and Gustafsson J, Interactions of indoles with specific binding sites for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rat liver. *Mol Pharmacol* **28:** 357–363, 1985.
30. Preobrazhenskaya MN, Korolev AM, Lazhko EI and Aleksandrova LG, Ascorbigen as a precursor of 5,11-dihydroindolo[3,2-*b*]carbazole. *Food Chem* **48:** 57–62, 1993.