FEBS Letters 520 (2002) 30–34 FEBS 26110

Identification of *o*-quinone/quinone methide metabolites of quercetin in a cellular in vitro system

Hanem M. Awad[a], Marelle G. Boersma[b], Sjef Boeren[a], Hester van der Woude[b], Jelmer van Zanden[b], Peter J. van Bladeren[b,c], Jacques Vervoort[a], Ivonne M.C.M. Rietjens[b,*]

[a] *Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands*
[b] *Division of Toxicology, Wageningen University, Tuinlaan 5, 6703 HE Wageningen, The Netherlands*
[c] *Nestle Research Centre, 1066 Vers-Chez-les Blanc, Switzerland*

Received 29 January 2002; revised 22 April 2002; accepted 22 April 2002

First published online 26 April 2002

Edited by Judit Ovádi

Abstract Formation of quercetin quinone/quinone methide metabolites, reflected by formation of the glutathionyl quercetin adducts as authentic metabolites, was investigated in an in vitro cell model (B16F-10 melanoma cells). Results of the present study clearly indicate the formation of glutathionyl quercetin adducts in a tyrosinase-containing melanoma cell line, expected to be representative also for peroxidase-containing mammalian cells and tissues. The data obtained also support that the adducts are formed intracellular and subsequently excreted into the incubation medium and reveal for the first time evidence for the pro-oxidative metabolism of quercetin in a cellular in vitro model.

Key words: Quercetin; Quinone/quinone methide; Melanoma cells; Glutathionyl adduct; Pro-oxidant

1. Introduction

Quinone and quinone methides from a variety of compounds, including 3′,4′-dihydroxyflavonoids, catechol-type metabolites from estrogens and polycyclic aromatic hydrocarbons, and compounds like the anticancer drug tamoxifen, have been classified as likely candidates for reactive metabolites able to react with cellular macromolecules [1–3]. For 3′,4′-dihydroxyflavonoids, with an intrinsic catechol moiety, their pro-oxidative quinone/quinone methide chemistry is especially of importance because of their increasing use as functional food ingredients and food supplements. Recently the glutathione (GSH) trapping method proved to be an excellent method to investigate the quinone/quinone methide chemistry of flavonoids [4–7], including quercetin, known to be mutagenic in a variety of bacterial and mammalian mutagenicity tests presumably through its quinone methide-like metabolites [8–10].

The actual formation of the glutathionyl-flavonoid quinone adducts and of their corresponding mercapturic acids in a cellular in vitro or in vivo system, would represent a bioactivation pathway of these supposed beneficial functional food ingredients, comparable to the formation of the glutathionyl and *N*-acetylcysteine conjugates of estrogens [11–15]. In addition, it has been suggested that the urinary levels of mercapturic acids can be used as a biomarker for exposure to active nucleophilic compounds pointing at possible risks for quinone-induced tumorigenesis [16–18]. Because the formation of these adducts would represent a toxic bioactivation pathway of supposed beneficial functional food ingredients, the detection of these quercetin glutathionyl adducts as authentic metabolites in cellular in vitro or in vivo models is of importance. As a first step in the search for the biological relevance of quinone methide-type pro-oxidant chemistry of flavonoids the objective of the present study was to investigate the possible formation of especially quercetin glutathione adducts as authentic metabolites in an in vitro cell model. The in vitro system used consisted of mouse melanoma cancer cells (B16F-10). These cells were chosen because they have been reported to contain significant levels of tyrosinase [19,20]. Tyrosinase, but also peroxidases, were shown before to catalyze flavonoid metabolism to quinone/quinone methide-type metabolites leading to glutathionyl-flavonoid adducts [4–7]. Thus, the B16F-10 melanoma cell line was considered an excellent model system to investigate the possible formation of quercetin glutathionyl adducts in cells exposed to quercetin.

2. Materials and methods

2.1. Materials

Quercetin was obtained from Acros Organics (NJ, USA). Glutathione, reduced form, and tyrosinase (EC 1.14.19.1) (from mushroom) were purchased from Sigma (St. Louis, MO, USA). All substrates were of 98–99% purity. Potassium hydrogen phosphate, potassium dihydrogen phosphate, citric acid, trisodium citrate dihydrate, anhydrous sodium carbonate, sodium hydrogen carbonate, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol were high performance liquid chromatography (HPLC) grade from Lab-Scan, Analytical Sciences (Dublin, Ireland).

2.2. Synthesis of quercetin quinone methide glutathione conjugates

To a starting solution of glutathione (final concentration of 1 mM) in 25 mM potassium phosphate pH 7.6 was added tyrosinase to a final concentration of 100 U/ml, followed by addition of 150 μM quercetin, added from a 10 mM stock solution in methanol. Upon 8 min incubation at 37°C, the incubation mixture was analyzed by HPLC.

2.3. Quercetin exposure and glutathionyl adduct formation

Mouse melanoma cancer cells (B16F-10) were obtained from ATCC (Manassas, VA, USA). B16F-10 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (obtained from

*Corresponding author. Fax: (31)-317-484801.
E-mail address: ivonne.rietjens@algemeen.tox.wau.nl (I.M.C.M. Rietjens).

0014-5793/02/$22.00
PII: S0014-5793(02)02754-0

Gibco BRL, Grand Island, NY, USA) with 25 mM HEPES, 4500 mg/l glucose, pyridoxine and without sodium pyruvate, supplemented with 10% fetal calf serum and 50 mg/l gentamicin (obtained from Gibco BRL), at 37°C in a humid atmosphere containing 5% CO_2. For each experiment, approximately 20×10^4 cells/ml were plated onto a 24-well tissue cluster (Costar, Cambridge, MA, USA) and cultured until a semi-confluent monolayer was obtained (in about 2 days). Cells were exposed to quercetin in quadruplicate for 1 h, 6 h, and 24 h at 37°C in (a) DMEM medium described above or (b) Hanks balanced salt solution (HBSS without phenol red and $NaHCO_3$, obtained from Gibco) to which $NaHCO_3$ was added to a final concentration of 0.35 g/l. The final volume of the medium was 0.5 ml. The concentration range of quercetin used throughout the experiments was 10, 25, 50, 75 and 100 μM. Quercetin was always added from a freshly prepared 200 times concentrated stock solution in DMSO. Exposure to quercetin was performed either in the absence or presence of vitamin C (final concentration of 1 mM) as indicated. Control incubations were included containing only DMSO at a final concentration of 0.5% in medium.

2.4. Analytical HPLC

HPLC was performed with a Waters M600 liquid chromatography system. Analytical separations were achieved using an Alltima C18 column (4.6×150 mm) (Alltech, Breda, The Netherlands). The column was eluted at 0.7 ml/min with water containing 0.1% (v/v) trifluoroacetic acid. A linear gradient from 10 to 30% acetonitrile in 12 min was applied, followed by 2 min isocratic elution with 30% acetonitrile. Hereafter a linear gradient from 30 to 100% acetonitrile was used in 2 min. The percentage of acetonitrile was kept at 100% for another 3 min. An injection loop of 10 μl was used. Detection was carried out with a Waters 996 photodiode array detector measuring spectra between 200 nm and 450 nm. Chromatograms presented are based on detection at 290 nm.

2.5. Liquid chromatography/mass spectrometry (LC/MS)

LC/MS analysis was performed to further characterize the peaks in the HPLC elution pattern. An injection volume of 10 μl from the incubation mixture was used and separation of the products was achieved on a 2.2×150 mm Alltima C18 column (Alltech, Breda, The Netherlands). A gradient from 10 to 30% acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid was applied at a flow of 0.2 ml/min in 13 min. The percentage of acetonitrile was kept at 30% for 2 min and then increased to 100% in another 2 min. Mass spectrometric analysis (LCQ 'classic', ThermoFinnigan, San Jose, CA, USA) was performed in the positive electrospray mode with the LCQ 'classic' electrospray interface using a spray voltage of 4.5 kV and a capillary temperature of 180°C with nitrogen as sheath and auxiliary gas.

3. Results

3.1. Incubation of quercetin with tyrosinase in the presence of glutathione

Fig. 1 shows the HPLC chromatogram of the incubation of quercetin with tyrosinase in the presence of GSH. Formation of two major adducts is observed which were previously identified as 6-glutathionyl and 8-glutathionyl quercetin hydrated in their C ring due to tyrosinase catalyzed water addition to the C2–C3 double bond in ring C [4,5,7]. Fig. 2a and b show the LC/MS for both metabolites which reveal the formation of an M+1 peak at m/z 626.0 for both metabolites and these LC/MS data, together with [^{1}H]NMR characteristics described previously [5,7] identify the nature of the metabolites as indicated in Fig. 1. The HPLC pattern presented in Fig. 1 is different from those shown in previous studies and reveals peaks which appear to be broader than in previous patterns because of different gradients. The gradient used in the present study appeared more suitable for detection of the glutathionyl quercetin adducts in cell line medium which contains several additional compounds. Also, the HPLC peaks of the GSH adducts broaden to an even further extent upon a decrease in the concentration (data not shown), as expected in the cellular incubations.

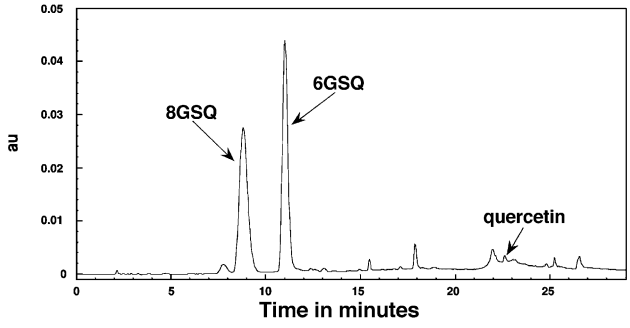

Fig. 1. HPLC chromatogram of the incubation of quercetin with tyrosinase in the presence of glutathione revealing the formation of 6-glutathionyl quercetin (6-GSQ) and 8-glutathionyl quercetin (8-GSQ).

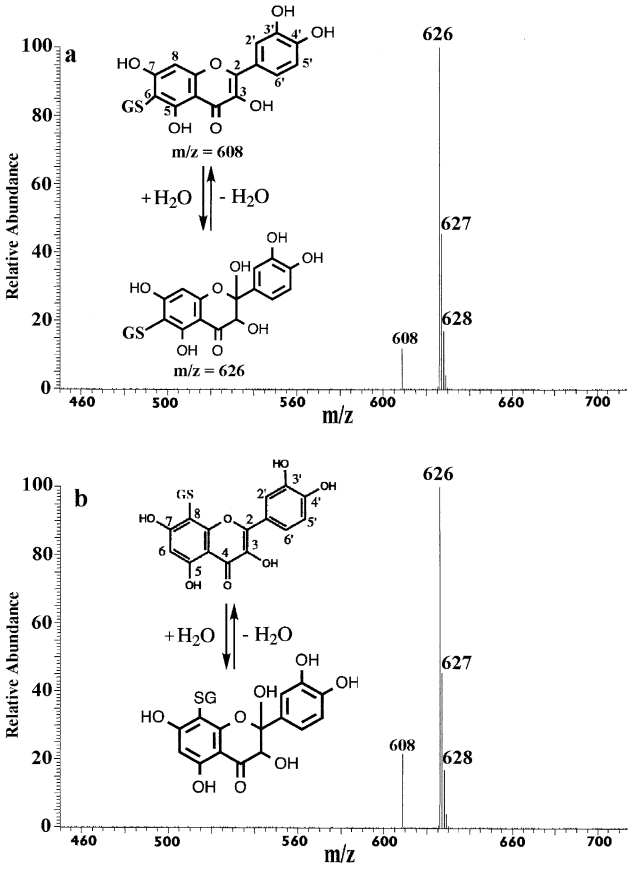

Fig. 2. Mass spectra of (a) 6-glutathionyl quercetin and (b) 8-glutathionyl quercetin revealing also the tyrosinase catalyzed H_2O addition to the glutathionyl quercetin adducts, which is in line with literature data [7,23]. The m/z values indicated are the values for the (M+1) forms of the structures indicated.

3.2. Quercetin quinone methide glutathione conjugate formation in a cellular in vitro model

Fig. 3a, b, and c show the HPLC chromatograms of DMEM of mouse B16F-10 melanoma cells exposed to 75 μM quercetin for 0 h, 1 h, and 6 h, respectively. Comparison of

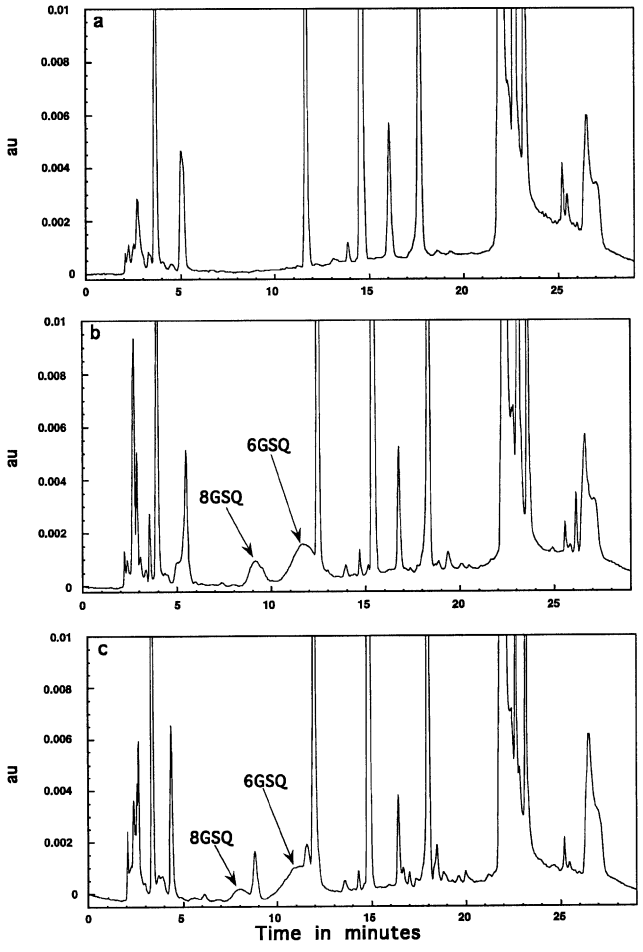

Fig. 3. HPLC chromatograms of DMEM of mouse B16F-10 melanoma cells exposed to 75 μM quercetin for (a) 0 h, (b) 1 h, and (c) 6 h.

Fig. 3b and c to Fig. 3a reveals the occurrence of two broad metabolite peaks with retention times of 9.1 and 11.4 min, which represent exactly the same retention times as those of 8- and 6-glutathionyl quercetin under the HPLC conditions applied (Fig. 1). The UV spectra of these two metabolites reveal an absorbance peak at 299.9 and 295.1 nm, respectively, and disappearance of the absorbance peak at 370.1 nm of quercetin indicating the loss of the conjugation between the A ring and B ring [21,22]. LC/MS analysis of these two metabolites reveals an M+1 peak at m/z 626 (Fig. 4a, b). This implies that retention times, UV spectra, and mass characteristics of these two metabolites are identical to those of 8- and 6-glutathionyl quercetin. Comparison of Fig. 3b to Fig. 3c reveals that the intensities of the two metabolite peaks decrease in time. After 24 h (HPLC chromatogram not shown), the presence of these two metabolites as well as of the parent quercetin were no longer observed indicating the instability of the metabolites and the full conversion of quercetin under the in vitro conditions used. The intracellularly formed GSH conjugates have been rapidly excreted into the medium since at the time of 1 h when glutathionyl quercetin concentrations were highest in the medium, no GSH conjugates could be detected by HPLC in the corresponding cellular samples (chromatogram not shown).

3.3. Quercetin glutathione conjugation in the presence of vitamin C

Fig. 5 presents the HPLC chromatogram pattern of medium of B16F-10 melanoma cells exposed to 75 μM quercetin for 1 h in the presence of vitamin C (final concentration of 1 mM). This experiment was performed to exclude the possible formation of the quercetin quinone/quinone methide and thus the 6- and 8-glutathionyl quercetin adducts due to chemical auto-oxidation of quercetin in the incubation medium. Comparison of the results presented in Fig. 3b to those in Fig. 5 reveals that both in the absence and presence of ascorbate formation of the 6- and 8-glutathionyl quercetin adducts was observed to a similar extent. This supports that the oxidation and glutathione conjugation are not due to an extracellular auto-oxidation process and can be ascribed to intracellular oxidation of quercetin by the tyrosinase present in the melanoma cells. Also the fact that the extracellular medium does not contain free GSH further supports that the 6- and 8-glutathionyl quercetin formation occurs intra- not extracellular, pointing at a true metabolic activation pathway.

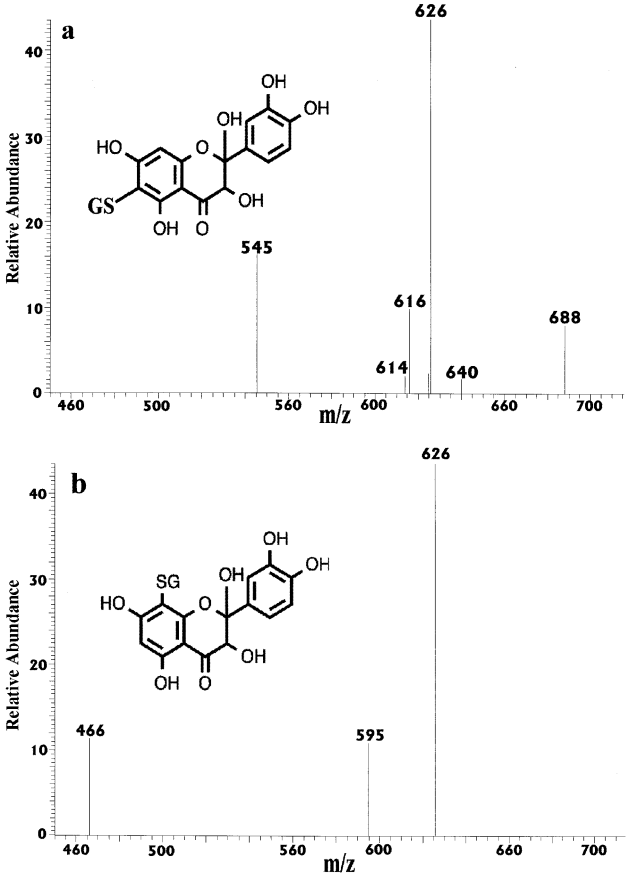

Fig. 4. Mass spectra of the two metabolites, (a) 6-GSQ and (b) 8-GSQ, detected in the DMEM of mouse B16F-10 melanoma cells during the exposure of 75 μM quercetin for 1 h. The m/z values indicated are the values for the (M+1) forms of the structures indicated.

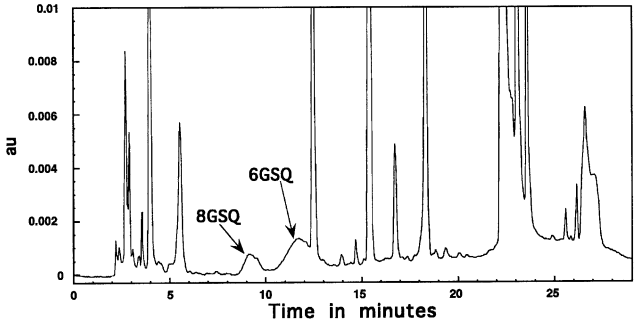

Fig. 5. HPLC chromatogram of DMEM of mouse B16F-10 melanoma cells exposed to 75 μM quercetin for 1 h in the presence of vitamin C (final concentration of 1 mM).

4. Discussion

Quinones represent a class of toxicological intermediates, which can create a variety of hazardous effects in vivo, including acute cytotoxicity, immunotoxicity and carcinogenesis [24,25]. The mechanisms by which quinones cause these effects can be quite complex. Quinones are Michael acceptors, and cellular damage can occur through alkylation of crucial cellular proteins and/or DNA. For 3′,4′-dihydroxyflavonoids, with an intrinsic catechol moiety, their pro-oxidant quinone/quinone methide chemistry is especially of importance because of their increasing use as functional food ingredients and food supplements. Therefore, the objective of the present study was to investigate the possible formation of the quinone/quinone methide metabolites of quercetin reflected by the formation of the glutathionyl quercetin adducts as authentic metabolites in an in vitro cell model. The cell model chosen consisted of B16F-10 melanoma cells known to contain significant amounts of tyrosinase [19,20]. The catechol-containing flavonoids have been shown to be substrates for tyrosinase and to deplete glutathione and protein thiols in melanoma cells. This effect might be ascribed to *o*-quinone formation although the actual formation of these *o*-quinones or of the glutathionyl conjugates derived from them was not demonstrated [19]. Results of the present study clearly indicate, for the first time, the formation of glutathionyl quercetin adducts in an in vitro cell model, providing direct evidence for the formation of quinone/quinone methide-type metabolites of the flavonoid model compound quercetin. The data obtained also support that the adducts are formed intracellular and subsequently excreted into the incubation medium.

Thus, the results of the present study reveal for the first time direct to be evidence for the pro-oxidative metabolism of quercetin in a cellular in vitro model, using the tyrosinase-containing B16F-10 melanoma cell line. However, not only tyrosinase but also peroxidases have been shown to be able to generate the quercetin quinone/quinone methide metabolites [4]. This implies that several mammalian cells and tissues known to contain peroxidase enzyme activities, including myeloperoxidase, lactoperoxidase, eosinophil peroxidase and thyroid peroxidase, may show the same formation of quercetin quinone/quinone methides as the tyrosinase-containing B16F-10 cells of the present study. Furthermore, peroxidase type of oxidation activity of polyphenols has been reported to be enhanced many-fold in response to estrogen agonists and may be related to pro-oxidative type of quinoid chemistry of polyphenols relevant for carcinogenesis [26]. Thus, the actual detection of quercetin quinone/quinone methide chemistry in the tyrosinase-containing B16F-10 melanoma cells of the present study, can be expected to have implications beyond the in vitro cell model used. Extension of the findings of the present study to peroxidase-containing mammalian cell types and tissues is an important topic for future research. Furthermore, the peroxidase-mediated formation of quercetin quinone/quinone methides indicates that upon one-electron oxidation of quercetin, the quercetin semiquinone radicals apparently rapidly disproportionate to give the two-electron oxidized quinone and the reduced parent quercetin. This suggests that quercetin quinoid-type metabolites may even be expected upon one-electron oxidation of quercetin upon its action as an anti-oxidant. If this would be the case the formation of quercetin quinoid-type metabolites could even be foreseen in all cellular systems in which quercetin acts as an electron donating anti-oxidant, although this also remains to be proven in future studies.

References

[1] Bolton, J.L., Pisha, E., Zhang, F. and Qiu, S. (1998) Chem. Res. Toxicol. 11, 1113–1126.
[2] Murty, V.S. and Penning, T.M. (1992) Chem. Biol. Interact. 84, 169–188.
[3] Zhang, F., Fan, P.W., Liu, X., Shen, L., van Breemen, R. and Bolton, J.L. (2000) Chem. Res. Toxicol. 13, 53–62.
[4] Awad, H.M., Boersma, M.G., Vervoort, J. and Rietjens, I.M.C.M. (2000) Arch. Biochem. Biophys. 378, 224–233.
[5] Boersma, M.G., Vervoort, J., Szymusiak, H., Lemanska, K., Tyrakowska, B., Cenas, N., Segura-Aguilar, J. and Rietjens, I.M.C.M. (2000) Chem. Res. Toxicol. 13, 185–191.
[6] Awad, H.M., Boersma, M.G., Boeren, S., Van Bladeren, P.J., Vervoort, J. and Rietjens, I.M.C.M. (2001) Chem. Res. Toxicol. 14, 398–408.
[7] Awad, H.M., Boersma, M.G., Boeren, S., Van Bladeren, P.J., Vervoort, J. and Rietjens, I.M.C.M. (2002) Chem. Res. Toxicol. 15, 343–351.
[8] Middleton, E., Jr. and Kandaswami, C. (1993) in: The Flavonoids: Advanced in Research Since 1986 (Harborne, J.H., Ed.), pp. 619–652, Chapman and Hall, New York.
[9] Murty, V.S. and Penning, T.M. (1992) Chem. Biol. Interact. 84, 169–188.
[10] MacGregor, J.T. and Jurd, L. (1978) Mutat. Res. 54, 297–309.
[11] Nakagomi, M. and Suzuki, E. (2000) Chem. Res. Toxicol. 13, 1208–1213.
[12] Zhu, B.T. and Conney, A.H. (1998) Carcinogenesis 19, 1–27.
[13] Cavalieri, E.L., Stack, D.E., Devanesan, P.D., Todorovic, R., Dwivedy, I., Higginbotham, S., Johansson, S.L., Patil, K.D., Gross, M.L., Gooden, J.K., Ramanathan, R., Cerny, R.L. and Rogan, E.G. (1997) Proc. Natl. Acad. Sci. USA 94, 10937–10942.
[14] Akanni, A. and Abul-Hajj, Y.J. (1997) Chem. Res. Toxicol. 10, 760–766.
[15] Liehr, J.G. (1997) Environ. Health Perspect. 105 (Suppl. 3), 565–569.
[16] Suzuki, E., Osabe, M., Okada, M., Ishizaki, T. and Suzuki, T. (1987) Jpn. J. Cancer Res. 78, 382–385.
[17] Vermeulen, N.P.E. (1989) Trends Pharmacol. Sci. 10, 177–181.
[18] Boogaard, P.J. and van Sittert, N.J. (1996) Environ. Health Perspect. 104, 1151–1157.
[19] Riley, P.A., Cooksey, C.J., Johnson, C.I., Land, E.J., Latter, A.M. and Ramsden, C.A. (1997) Eur. J. Cancer 33, 135–143.
[20] Ishiguro, S.I., Yamamoto, H., Yanai, N. and Takeuchi, T. (1986) J. Biochem. 99, 1081–1085.
[21] Galati, G., Moridani, M.Y., Chan, T.S. and O'Brien, P.J. (2001) Free Radic. Biol. Med. 30, 370–382.

[22] Howard, P.H., Arlend, D.K. and Craig, E.L. (1994) J. Pharmacol. Biol. Anal. 12, 325–331.
[23] Frey-Schröder, G. and Barz, W. (1979) Z. Naturforsch. 34c, 200–209.
[24] Bolton, J.L., Trush, M.A., Penning, T.M., Dryhurst, G. and Monks, T.J. (2000) Chem. Res. Toxicol. 13, 135–160.
[25] Bolton, J.L., Pisha, E., Zhang, F. and Qiu, S. (1998) Chem. Res. Toxicol. 11, 1113–1126.
[26] Daly, B.W., Tyurin, V.A., Tyurina, Y.Y., Liu, M., Facey, J.A., Carta, G., Kisin, E.R., Dubey, R.K. and Kagan, V.E. (1999) Chem. Res. Toxicol. 12, 28–37.