REFERENCES

1 Hosokawa M. Structure and catalytic properties of carboxylesterase isozymes involved in metabolic activation of prodrugs. Molecules 2008; 13: 412–31.

2 Humerickhouse R, Lohrbach K, Li L, Bosron WF, Dolan ME. Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. Cancer Res 2000; 60: 1189–92.

3 Ando Y, Saka H, Ando M, Sawa T, Muro K, Ueoka H, Yokoyama A, Saitoh S, Shimokata K, Hasegawa Y. Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. Cancer Res 2000; 60: 6921–6.

4 Iyer L, Das S, Janisch L, Wen M, Ramirez J, Karrison T, Fleming GF, Vokes EE, Schilsky RL, Ratain MJ. UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity. Pharmacogenomics J 2002; 2: 43–7.

5 Innocenti F, Undevia SD, Iyer L, Chen PX, Das S, Kocherginsky M, Karrison T, Janisch L, Ramírez J, Rudin CM, Vokes EE, Ratain MJ. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. J Clin Oncol 2004; 22: 1382–8.

6 Han JY, Lim HS, Shin ES, Yoo YK, Park YH, Lee JE, Jang IJ, Lee DH, Lee JS. Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin. J Clin Oncol 2006; 24: 2237–44.

7 Minami H, Sai K, Saeki M, Saito Y, Ozawa S, Suzuki K, Kaniwa N, Sawada J, Hamaguchi T, Yamamoto N, Shirao K, Yamada Y, Ohmatsu H, Kubota K, Yoshida T, Ohtsu A, Saijo N. Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. Pharmacogenet Genomics 2007; 17: 497–504.

8 Sai K, Saito Y, Sakamoto H, Shirao K, Kurose K, Saeki M, Ozawa S, Kaniwa N, Hirohashi S, Saijo N, Sawada J, Yoshida T. Importance of UDP-glucuronosyltransferase 1A1*6 for irinotecan toxicities in Japanese cancer patients. Cancer Lett 2008; 261: 165–71.

9 Sai K, Kaniwa N, Itoda M, Saito Y, Hasegawa R, Komamura K, Ueno K, Kamakura S, Kitakaze M, Shirao K, Minami H, Ohtsu A, Yoshida T, Saijo N, Kitamura Y, Kamatani N, Ozawa S, Sawada J. Haplotype analysis of ABCB1/MDR1 blocks in a Japanese population reveals genotype-dependent renal clearance of irinotecan. Pharmacogenetics 2003; 13: 741–57.

10 Sai K, Saito Y, Maekawa K, Kim SR, Kaniwa N, Nishimaki-Mogami T, Sawada J, Shirao K, Hamaguchi T, Yamamoto N, Kunitoh H, Ohe Y, Yamada Y, Tamura T, Yoshida T, Matsumura Y, Ohtsu A, Saijo N, Minami H. Additive effects of drug transporter genetic polymorphisms on irinotecan pharmacokinetics/pharmacodynamics in Japanese cancer patients. Cancer Chemother Pharmacol 2010; 66: 95–105.

11 Han JY, Lim HS, Park YH, Lee SY, Lee JS. Integrated pharmacogenetic prediction of irinotecan pharmacokinetics and toxicity in patients with advanced non-small cell lung cancer. Lung Cancer 2009; 63: 115–20.

12 Innocenti F, Kroetz DL, Schuetz E, Dolan ME, Ramírez J, Relling M, Chen P, Das S, Rosner GL, Ratain MJ. Comprehensive pharmacogenetic analysis of irinotecan neutropenia and pharmacokinetics. J Clin Oncol 2009; 27: 2604–14.

13 Kubo T, Kim SR, Sai K, Saito Y, Nakajima T, Matsumoto K, Saito H, Shirao K, Yamamoto N, Minami H, Ohtsu A, Yoshida T, Saijo N, Ohno Y, Ozawa S, Sawada J. Functional characterization of three naturally occurring single nucleotide polymorphisms in the CES2 gene encoding carboxylesterase 2 (HCE-2). Drug Metab Dispos 2005; 33: 1482–7.

14 Kim SR, Sai K, Tanaka-Kagawa T, Jinno H, Ozawa S, Kaniwa N, Saito Y, Akasawa A, Matsumoto K, Saito H, Kamatani N, Shirao K, Yamamoto N, Yoshida T, Minami H, Ohtsu A, Saijo N, Sawada J. Haplotypes and a novel defective allele of CES2 found in a Japanese population. Drug Metab Dispos 2007; 35: 1865–72.

15 Tanimoto K, Kaneyasu M, Shimokuni T, Hiyama K, Nishiyama M. Human carboxylesterase 1A2 expressed from carboxylesterase 1A1 and 1A2 genes is a potent predictor of CPT-11 cytotoxicity in vitro. Pharmacogenet Genomics 2007; 17: 1–10.

16 Fukami T, Nakajima M, Maruichi T, Takahashi S, Takamiya M, Aoki Y, McLeod HL, Yokoi T. Structure and characterization of human carboxylesterase 1A1, 1A2, and 1A3 genes. Pharmacogenet Genomics 2008; 18: 911–20.

17 Hosokawa M, Furihata T, Yaginuma Y, Yamamoto N, Watanabe N, Tsukada E, Ohhata Y, Kobayashi K, Satoh T, Chiba K. Structural organization and characterization of the regulatory element of the human carboxylesterase (CES1A1 and CES1A2) genes. Drug Metab Pharmacokinet 2008; 23: 73–84.

18 Geshi E, Kimura T, Yoshimura M, Suzuki H, Koba S, Sakai T, Saito T, Koga A, Muramatsu M, Katagiri T. A single nucleotide polymorphism in the carboxylesterase gene is associated with the responsiveness to imidapril medication and the promoter activity. Hypertens Res 2005; 28: 719–25.

19 Yoshimura M, Kimura T, Ishii M, Ishii K, Matsuura T, Geshi E, Hosokawa M, Muramatsu M. Functional polymorphisms in carboxylesterase1A2 (CES1A2) gene involves specific protein 1 (Sp1) binding sites. Biochem Biophys Res Commun 2008; 369: 939–42.

20 Kitamura Y, Moriguchi M, Kaneko H, Morisaki H, Morisaki T, Toyama K, Kamatani N. Determination of probability

distribution of diplotype configuration (diplotype distribution) for each subject from genotypic data using the EM algorithm. Ann Hum Genet 2002; 66: 183–93.

21 Sai K, Saito Y, Fukushima-Uesaka H, Kurose K, Kaniwa N, Kamatani N, Shirao K, Yamamoto N, Hamaguchi T, Kunitoh H, Ohe Y, Tamura T, Yamada Y, Minami H, Ohtsu A, Yoshida T, Saijo N, Sawada J. Impact of CYP3A4 haplotypes on irinotecan pharmacokinetics in Japanese cancer patients. Cancer Chemother Pharmacol 2008; 62: 529–37.

22 Charasson V, Bellott R, Meynard D, Longy M, Gorry P, Robert J. Pharmacogenetics of human carboxylesterase 2, an enzyme involved in the activation of irinotecan into SN-38. Clin Pharmacol Ther 2004; 76: 528–35.

23 Marsh S, Xiao M, Yu J, Ahluwalia R, Minton M, Freimuth RR, Kwok PY, McLeod HL. Pharmacogenomic assessment of carboxylesterases 1 and 2. Genomics 2004; 84: 661–8.

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RESEARCH ARTICLE

DNA methylation and its involvement in *carboxylesterase 1A1* (*CES1A1*) gene expression

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Abstract

1. Carboxylesterase 1A1 (CES1A1) efficiently catalyses the hydrolysis of a substrate containing ester, amide, or thioester bonds. It is expressed at a high level in the human liver, but at a low level in the human kidney. In this study, we found the cause of this tissue-specific expression of the *CES1A1* gene using 5-aza-2′-deoxycytidine (5-aza-dC) and bisulfite sequencing.

2. Treatment of HEK293 cells, human embryonic kidney cells not expressing the *CES1A1* gene, with 5-aza-dC caused dramatic expression of the *CES1A1* gene. Bisulfite sequencing revealed that the region around the transcription start site (TSS) of the *CES1A1* gene was almost entirely methylated in HEK293 cells, whereas the region was almost entirely unmethylated in HepG2 cells, human hepatoma cells. The hypomethylated DNA molecules for the region were observed in HEK293 cells treated with 5-aza-dC. In the genome obtained from the kidney, the region downstream of the TSS was methylated compared with that obtained from the liver.

3. From these findings, it can be concluded that DNA methylation is involved in *CES1A1* gene expression and that the difference between *CES1A1* gene expression in the human kidney and that in the human liver may arise from the difference in DNA methylation levels in the region around the TSS.

Keywords: *Tissue-specific expression; CpG island; prodrug; oseltamivir*

Introduction

Mammalian carboxylesterases (CESs, EC 3.1.1.1) are encoded by a multigene family (Satoh et al. 2002) and are members of an α,β-hydrolase-fold family (Bencharit et al. 2003). CESs are ubiquitously expressed, particular in the liver, small intestine, kidney and lung, in various mammals, and the majority of CESs are localized to the endoplasmic reticulum (Satoh et al. 2002). CESs efficiently catalyse the hydrolysis of a substrate containing ester, amide, or thioester bonds. Therefore, they play an important role in the metabolic activation of prodrugs that are designed to improve bioavailability. According to the homology of amino acid sequence,

we previously classified CESs into five major groups, CES1–5 (Satoh & Hosokawa 2006), and found that many of the identified CESs belong to the CES1 or CES2 family. The substrate specificities of the CES1 and CES2 families are very different. The CES1 isozyme mainly hydrolyses a substrate with a small alcohol group and large acyl group. In contrast, the CES2 isozyme mainly hydrolyses a substrate with a large alcohol group and small acyl group. The CES1 family can be divided into eight subfamilies (CES1A-H). CES1A1 is a human CES1A subfamily isozyme and is mainly expressed in the liver and lung. CES2A1 is a human CES2A subfamily isozyme and is mainly expressed in the small intestine and kidney (Hosokawa 2008).

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CESs are related to transporters and conjugation enzymes and they are involved in drug metabolism and disposition. For instance, CESs convert temocapril, an angiotensin-converting enzyme inhibitor, to the active metabolite temocaprilate, which is transported by a canalicular multispecific organic anion transporter (cMOAT, ABCC2) (Ishizuka et al. 1997). Furthermore CESs hydrolyse irinotecan hydrochloride (CPT-11), an anti-tumour drug, to the active metabolite SN-38, which is a good substrate for UDP-glucuronosyltransferase (UGT) (Sanghani et al. 2004).

It is important to understand the tissue-specific expression of CESs for design of ideal prodrugs that are efficiently hydrolysed in the target tissue and are associated with sufficient drug efficacy and few side-effects. The tissue-specific expression of CESs in the human kidney is involved in renal excretion, since CESs change the polarity of a prodrug. As mentioned above, CES2A1 is expressed in the human kidney. Therefore, it participates in renal excretion of a prodrug. Other drug-metabolizing enzymes that are expressed in the human kidney also play an important role in the metabolism of exogenous and endogenous compounds. For example, the glucuronidation of propofol is catalysed by UGT in the kidney (McGurk et al. 1998) and the secosteroid hormone 1,25-dihydroxyvitamin D3 (Calcitriol) is catalysed by 25-hydroxyvitamin D3 1-alpha-hydroxylase (CYP27B1) in the kidney (Zehnder et al. 1999). In this way, many drug-metabolizing enzymes expressed in the human kidney contribute to the metabolism of compounds. However, the CES1A1 gene is poorly expressed in the human kidney. We previously reported that the transcription factor specificity protein (Sp) 1, which is ubiquitously expressed, can bind to the promoter region of the CES1A1 gene, leading to transactivation of the promoter (Hosokawa et al. 2008). Hence, we have thought that there is a mechanism repressing CES1A1 gene expression in the human kidney.

On the other hand, gene regulation by DNA methylation has been reported for some drug-metabolizing enzymes (Anttila et al. 2003; Gagnon et al. 2006). In mammals, DNA methylation occurs predominantly on cytosine in 5'-CpG-3' dinucleotide and its methylation mark is propagated into both DNA strands after DNA replication. Approximately 40% of mammalian genes include CpG islands, regions with relatively high frequency of CpG nucleotides, in their promoters and exonic regions (Larsen et al. 1992). It is generally accepted that DNA methylation of a CpG island at a promoter region is closely associated with silencing of a gene expression. It is also known that DNA methylation in the region downstream of a transcription start site (TSS) causes dramatic reduction in gene expression (Graessmann et al. 1994; Hisano et al. 2003). Recently, it was proposed that a DNA methylation-free region

extending several hundred bases downstream of the TSS may be a prerequisite for efficient transcription initiation (Appanah et al. 2007). The tissue-specific expression of a number of genes has been revealed by studies on DNA methylation (Kikuchi et al. 2007; Aoki et al. 2008).

The aim of the present study was to elucidate the cause of tissue-specific expression of the CES1A1 gene, particularly the cause of the difference in gene expression in the human kidney and liver.

Materials and methods

Cell lines and human tissues

HEK293 cells, human embryonic kidney cells, and HuH-7 and HepG2 cells, human hepatoma cells, were used in this study. CES1A1 gene is not expressed in HEK293 cells but is expressed in HuH-7 and HepG2 cells. The cell lines were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) foetal bovine serum and penicillin/streptomycin. Human kidney and liver were obtained from the National Disease Research Interchange (Philadelphia, PA, USA) through the Human and Animal Bridging Research Organization (HAB) (Chiba, Japan).

Treatment with DNA methylation inhibitor and histone deacetylase inhibitor

HEK293, HuH-7, and HepG2 cells were precultured for 24 h and then cultured for 3 days in medium containing 2 µM 5-aza-2'-deoxycytidine (5-aza-dC), a DNA methylation inhibitor (Sigma-Aldrich, St. Louis, MO, USA), diluted with PBS(-). Subsequently, total RNA was extracted. For histone deacetylase inhibition, 500 nM trichostatin A (TSA) (Wako, Osaka, Japan) diluted with ethanol was added to the cells 24 h before extraction of RNA.

Relative quantification of mRNA by real-time polymerase chain reaction (PCR)

Total RNA was extracted from the cell lines and tissues using an ISOGEN (Nippon Gene, Toyama, Japan) and then treated with DNase I (Invitrogen). Subsequently, cDNA was synthesized with the RNA using a ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Finally, the expression level of CES1A1 mRNA was analysed using a Realtime PCR Master mix (Toyobo) with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster, CA). The specific primers were designed as follows: forward, 5'-GAGACCTCGCAGGCCCC-3'; reverse,

5′-GACGAACTTCCCCAGCACTT-3′. The fluorescent probe for CES1A1 was 5′-(FAM)-TCCGTGCCTTTATC-3′. Real-time PCR was performed under the following conditions: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 56°C for 1 min. The CES1A1 mRNA expression was normalized with 18S rRNA expression:

Delta C_t (dC_t) = threshold cycle (C_t) for target amplification − C_t for reference amplification

In each cell line, the delta delta C_t (ddC_t) values were based on the average dC_t value of the cell line treated with 5-aza-dC alone, except that the ddC_t value of HuH-7 cells for Figure 1 was based on the average dC_t value of HEK293 cells treated with 5-aza-dC. The average mRNA expression ratio of three liver specimens was indicated as the result for the liver. The mRNA expression ratio of a specimen in which CES1A1 mRNA was always detected was selected as the representative value for the kidney. All assays were performed in triplicate. For improvement of the assay of CES1A1 mRNA used in our previous study (Hosokawa et al. 2008), CES1A1 wild-type gene and a CES1A1 gene variant were distinguished by sequencing and tissues that have only CES1A1 wild-type gene were used in the present study. The CES1A1 gene variant has the same exon 1 as that of the CES1A3 gene instead of that of the CES1A1 wild-type gene (Tanimoto et al. 2007; Fukami et al. 2008).
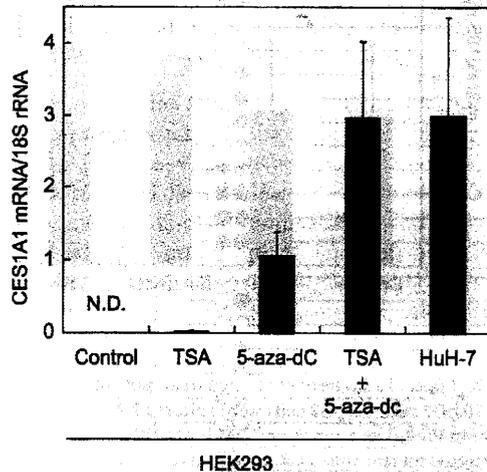

Figure 1. Effect of a single or combined treatment with 5-aza-dC and TSA in HEK293 cells. HEK293 cells were cultured for 3 days in medium containing PBS(−) as a mock or 2 μM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 500 nM TSA was added to the cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean ± standard deviation (SD) of three independent experiments, which were performed in triplicate. Results for HuH-7 cells are shown for comparison with results for HEK293 cells treated with 5-aza-dC. N.D., The mRNA was not detected.

Determination of the CpG island

A search for the CpG island of the CES1A1 gene was carried out using GENETYX-Mac Ver.12.2.0 software. The CpG island was determined using MethPrimer online software (Li & Dahiya 2002). The criteria used were island size > 200 bp, GC % > 50.0, and observed/expected CpG ratio > 0.6.

Bisulfite sequencing

Genomic DNA was extracted from the human kidney, human liver, HEK293 cells, and HepG2 cells using a FastPure™ DNA Kit (Takara Bio, Shiga, Japan). After purification, the genomic DNA was treated with sodium bisulfite using an Epitect™ Bisulfite Kit (Qiagen, Hilden, Germany). The target region (−512 to + 214) in the CES1A1 gene was amplified from the genomic DNA by PCR using a GoTaq® Green Master Mix (Promega, Madison, WI, USA) and primers designed as follows: forward, 5′-TTGTGAAGTTAATTTAGGTTTTAGAAAGG-3′; reverse, 5′-AACCTATTATATCTTTACCTTTCTAC-3′. PCR was performed under the following conditions: 95°C for 2 min; 40 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and finally 72°C for 5 min. To perform hot start PCR, the reverse primer was added to the reaction mix when the reaction mix first reached 95°C. After being purified, the PCR products were cloned into a pGEM-T vector (Promega). Approximately ten clones for each tissue specimen were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a Montage™ SEQ$_{96}$ Sequencing Reaction Cleanup Kit (Millipore, Billerica, MA, USA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). In addition, each of approximately 30 clones obtained from three independent experiments were sequenced for HEK293 cells and HEK293 cells treated with 5-aza-dC, and approximately 20 clones obtained from two independent experiments were sequenced for HepG2 cells. CpG methylation status was analysed by the web-based tool QUMA (Kumaki et al. 2008). Three human kidney specimens (age/sex, 47/male, 54/male, and 75/female) and three human liver specimens (age/sex, 62/female, 68/male, and 70/female) were used. Three TSSs of the CES1A1 gene are known and the most upstream TSS was selected as TSS (+ 1) in the present study.

Enzyme assay

The activity of the hydrolysis for 0.1 mM p-nitrophenyl acetate (PNPA) was determined colorimetrically according to the method of Hosokawa et al. (1987). The cell culture and the treatment with 5-aza-dC and TSA were performed at the same condition of mRNA experiment

described above. Three independent experiments were performed.

Statistical analysis

All data of mRNA expression ratios were tested by the Smirnov–Grubbs test ($p < 0.01$) and two extreme values were rejected. The results of bisulfite sequencing at each CpG site for the human kidney and liver were tested by Fisher's exact test, and that of the entire set of CpG sites for the human kidney and liver were tested by the Mann–Whitney U-test using the web-based tool QUMA (Kumaki et al. 2008). The results of enzyme assay were tested by Student's t-test.

Results

Comparison of CES1A1 mRNA expression levels in the human kidney, liver and the cell lines

We confirmed that the expression level of CES1A1 mRNA in the human liver is much higher than that in the kidney and that CES1A1 mRNA is detected in HuH-7 and HepG2 cells. When the ddC_t values of the kidney, liver and the cell lines were based on the average dC_t value of HuH-7 cells, the mRNA expression ratio (CES1A1 mRNA/18S rRNA) of the kidney, HuH-7, HepG2, and the liver was 0.106 ± 0.0223, 1.11 ± 0.508, 29.9 ± 14.4 and 76.2 ± 96.9 (mean $\pm$ standard deviation (SD)), respectively.

Activation of the CES1A1 gene in HEK293 cells by 5-aza-dC

CES1A1 mRNA was not detected in HEK293 cells by real-time PCR (Figure 1). To investigate whether the CES1A1 gene in HEK293 cells is silenced by DNA methylation, we treated HEK293 cells with 2 µM 5-aza-dC for 3 days. As a result, we detected dramatic expression of CES1A1 mRNA in HEK293 cells (Figure 1). In addition, 500 nM TSA enhanced the expression in HEK293 cells treated with 5-aza-dC, but single treatment with TSA had little effect on mRNA expression in HEK293 cells. CES1A1 mRNA expression in HEK293 cells treated with 5-aza-dC and TSA was almost the same as the expression in HuH-7 cells alone. Furthermore, the dramatic enhancement of CES1A1 mRNA expression observed in HEK293 cells treated with 5-aza-dC was not observed in HuH-7 and HepG2 cells treated with 5-aza-dC (Figures 1–3).

Negative correlation between methylation and expression of the CES1A1 gene

To determine the methylation status of the CES1A1 gene in the human kidney and liver and the cell lines,
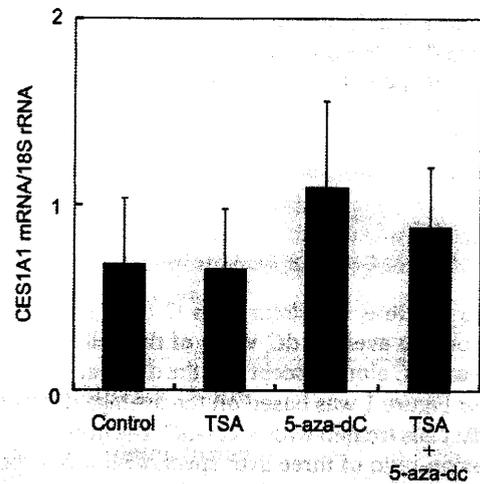

Figure 2. Effect of a single or combined treatment with 5-aza-dC and TSA in HuH-7 cells. HuH-7 cells were cultured for 3 days in medium containing PBS(-) as a mock or 2 µM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 500 nM TSA was added to the cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean $\pm$ standard deviation (SD) of three independent experiments, which were performed in triplicate.
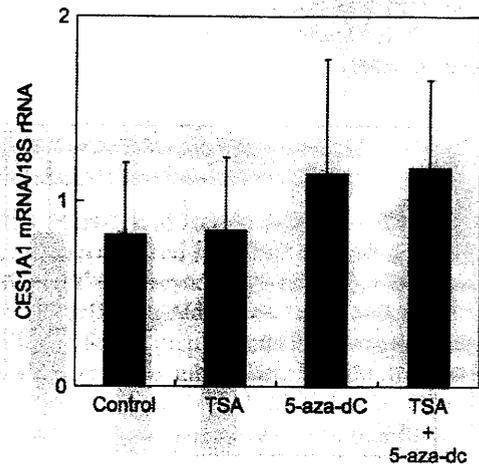

Figure 3. Effect of a single or combined treatment with 5-aza-dC and TSA in HepG2 cells. HepG2 cells were cultured for 3 days in medium containing PBS(-) as a mock or 2 µM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 500 nM TSA was added to the cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean $\pm$ standard deviation (SD) of four independent experiments, which were performed in triplicate.

we performed bisulfite sequencing for the region of 726 bp (−512 to +214) (Figure 4). The results revealed that the region around the TSS of the CES1A1 gene is almost entirely methylated in HEK293 cells, whereas the region is almost entirely unmethylated in HepG2 cells (Figure 5). As a result of treatment with 2 µM

-512 TTGTGAAGCTAATTCAGGCCTCAGAAAGGGGACT CG ATGAAATTTAAGAT CG CTTCCAAGCTTGAGAGCCTGGAAAGCTATGA

-429 AAACACAAGCCCTGGGAGCTGAGATATGTCCTAACTTACCCAGCTGAGCTGTGAGGTGTGAGTGGCTCTAACATTTTCCAGTTG

-345 TTTCTGAGGACCTCAGATCAAAGCTTCCCTTTGCCTAAAAGCATCTGCCTGCTGGTGTGGGCCCTTGGGGGC CG TCACAGTGCA

-261 CTGAGGTTAGAGTCCTGCAAGGGTGAACCCTTATGTAACAAGTAGTTGGGCAAGTTTACAGCTCTCTGTAATCTGACAGTAGAG

-177 TCCAGACTGGTTTGATGAAAGAGGGTAAACTGTGGGTGGG CG TGGCCTGAGGCCCCACTAGAAGCCCAGGGAGATCTGAGGAA

-94 AGGGAGGGCTTTTCTGATCTCTCCCAATTAGAGGATTAGGCAATTGGCAGC CG CAGGC CG GTAACTCTGGGC CG GGGCTGGGCTC

-11 CAGGGCTGGAC A GCACAGTGCCTCTGAACTGCACAGAGACCT CG CAGGCCCT CG GAGAACTGT CG CCCTTCCAC CG ATCTGGCTC
          +1

+72 CG TGCCTTTATCC TGGCCACTCTCTCTGCTTC CGC GG GCTTGGG GTGAGTCCTTCTGAAATCAAATATGC CG GGGCACTTTTTGAAA

+157 TCCTTGTTCTGGGC CG AGGTGGGC CG CAGATGC CG TAGAAAGGCAAAGACACAACAGGTC

Figure 4. Positions of CpGs in the region of 726 bp (–512 to + 214) around the TSS of the *CES1A1* gene. Positions of CpGs are shown in boxes. A broken arrow with a box indicates TSS (+ 1). Exon 1 is shown in a shaded box. A part of the CpG island is underlined with a heavy line. ATG is underlined with a thin line.
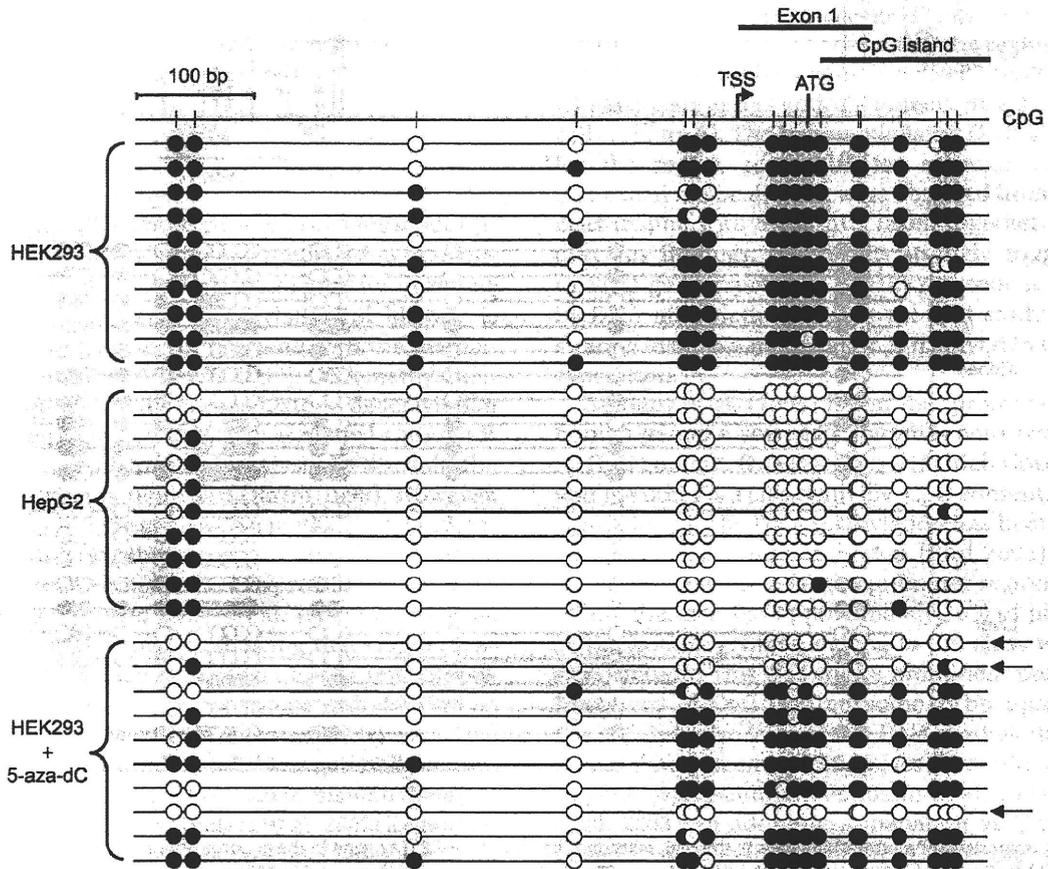

Figure 5. Appearance of hypomethylated molecules for the region around the TSS of the *CES1A1* gene in HEK293 cells treated with 5-aza-dC. Bisulfite sequencing was performed to determine the methylation pattern in the region from –512 to + 214. A broken arrow indicates TSS (+ 1). Each CpG position is depicted as a vertical bar. The methylated and unmethylated CpGs are depicted as closed circles and open circles, respectively. Ten representative molecules of each cell line are shown. Horizontal arrows indicate hypomethylated molecules.

5-aza-dC for 3 days, hypomethylated DNA molecules for the region accounted for approximately 30% of the total molecules that were obtained from HEK293 cells treated with 5-aza-dC. In the genome obtained from the kidney, the region downstream of the TSS was methylated compared with that obtained from the liver, although the difference in methylation levels between the kidney and liver was less clear than that
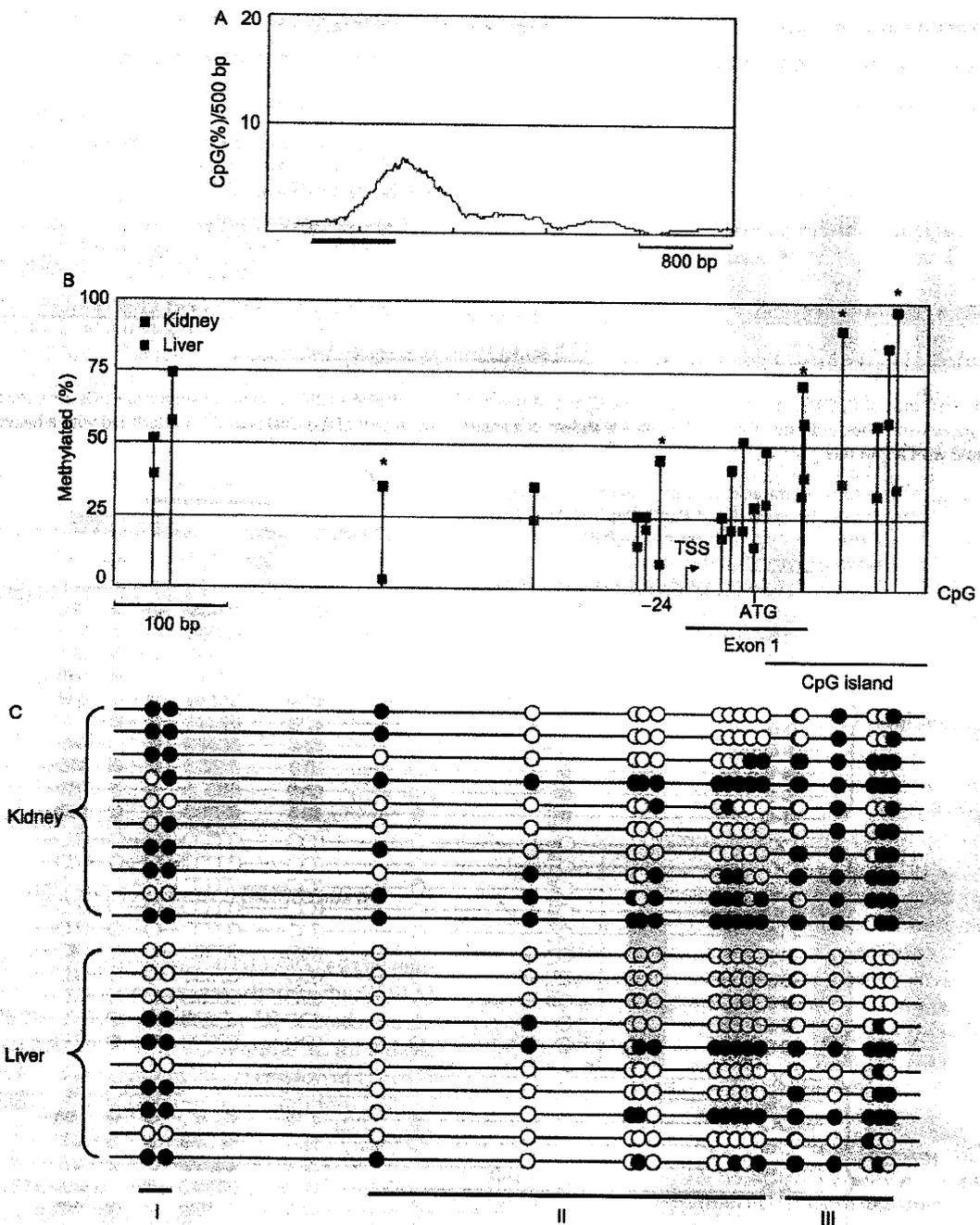
Figure 6. Comparison of methylation levels in the region around the TSS of the *CES1A1* gene in the genome obtained from the human kidney and liver. (A) CpG island (+72 to +541) of the *CES1A1* gene. The CpG content (%) was computed in 500 bp overlapping segments across a 4-kb region. A heavy line indicates the amplified region of 726 bp. (B) Bisulfite sequencing was performed to determine the methylation pattern of the kidney and liver in the region of 726 bp (-512 to +214). The methylated levels (%) of the kidney and liver are shown with the TSS (broken arrow), ATG, exon 1, and a part of the CpG island (+72 to +214). The graph was made using approximately 30 molecules of each tissue. Each CpG position is depicted as a vertical bar. Asterisks indicate statistically significant differences in the methylation level between the human kidney and liver ($p < 0.01$, Fisher's exact test). There was the statistically significant difference in the methylation level of the entire set of CpG sites between the kidney and liver ($p < 0.01$, Mann–Whitney U-test). (C) The methylated and unmethylated CpGs are depicted as closed circles and open circles, respectively. Ten representative molecules of each tissue are shown.

between the cell lines (Figure 6). There was little difference in the methylation among individual liver or kidney specimens.

Activity of the hydrolysis of p-nitrophenyl acetate (PNPA)

As the results of the activity caused by single or combined treatment with 5-aza-dC and TSA to HEK293 cells, the values (nmol 10^{-6} cells min^{-1}) of control, TSA, 5-aza-dC, and TSA + 5-aza-dC were 1.67 ± 0.425 (1.00-fold), 1.98 ± 0.377 (1.18-fold), 2.10 ± 0.0528 (1.26-fold), and 2.21 ± 0.680 (1.32-fold) (mean ± SD), respectively. But statistically significant differences were not observed in the data of the activity of HEK293 cells. The result of the activity of HuH-7 cells was 13.3 ± 1.95 nmol 10^{-6} cells min^{-1}. Correlation coefficient between PNPA hydrolase activity and mRNA expression level caused by single or combined treatment with 5-aza-dC and TSA to HEK293 cells was 0.789.

Discussion

DNA methylation is closely related to histone modifications such as deacetylation and methylation in mammals (Cameron et al. 1999). Histone proteins assemble into nucleosomes, and deacetylation of histone is important for silencing of the gene with the methylated promoter (Bird & Wolffe 1999). DNA methylation in the promoter region causes histone deacetylation by mediating some proteins such as methyl-CpG binding protein and histone deacetylase. As a result, the gene expression is repressed (Razin 1998). However, once silencing of the gene with a methylated promoter is established, inhibition of histone deacetylation generally cannot cause active expression of the gene (Cameron et al. 1999; Coffee et al. 1999; El-Osta et al. 2002). The present results showing that single treatment with TSA had little effect on *CES1A1* gene expression in HEK293 cells are consistent with the finding described above (Figure 1). The reason why treatment with a histone deacetylase inhibitor generally cannot cause active expression is related to histone methylation as described below (Peters et al. 2002; Zegerman et al. 2002). According to Kondo et al. (2003), methylation on lysine 9 (Lys-9) in histone H3 causes a repressive folded chromatin structure, affects the access of regulatory factors to chromatin, and leads to silencing of *P16*, *MLH1*, and *MGMT* genes in colorectal cancer. Treatment with 5-aza-dC decreased DNA methylation and Lys-9 methylation dramatically, increased Lys-9 acetylation slightly and Lys-4 methylation moderately, and reactivated gene expression (Kondo et al. 2003). Consistent with these findings obtained by treatment

with 5-aza-dC, our results showed that treatment with 5-aza-dC allows HEK293 cells to activate *CES1A1* gene expression (Figure 1). Combined treatment with 5-aza-dC and TSA decreased DNA methylation and Lys-9 methylation and increased Lys-9 acetylation markedly and Lys-4 methylation, while single treatment with TSA increased Lys-9 acetylation and had no effect on Lys-9 or Lys-4 methylation (Kondo et al. 2003). The acetylation level of Lys-9 in the case of combined treatment was higher than that in the case of treatment with 5-aza-dC alone. Consequently, a high expression level of the gene was observed. In agreement with these findings about combined treatment, the results showed that the level of *CES1A1* gene expression induced by combined treatment with 5-aza-dC and TSA was approximately three times higher than that induced by treatment with 5-aza-dC alone in HEK293 cells (Figure 1). The results of bisulfite sequencing showed that the region around the TSS of the *CES1A1* gene is entirely methylated in HEK293 cells and is entirely unmethylated in HepG2 cells (Figure 5). The hypomethylated DNA molecules for the region accounted for approximately 30% of the total molecules that were obtained from HEK293 cells treated with 5-aza-dC. Taken together with the previous findings, our results strongly suggest that *CES1A1* gene expression in HEK293 cells is silenced by DNA methylation. This is the first study demonstrating that DNA methylation is involved in *CES* gene expression.

Saxonov et al. (2006) found that promoters in the human genome segregate naturally into two classes by CpG content. One is a class with high CpG content and the other is a class with low CpG content. To date, gene silencing by DNA methylation has been studied mainly for the promoter region (Bird 2002). Studies on DNA methylation for the promoter region seem to reflect the fact that approximately 70% of promoters in the human genome belong to the class with high CpG content. This class has a prominent peak in the frequency of CpG centred some 15 bp upstream of the TSS. It is also known that DNA methylation in the region downstream of TSS causes dramatic reduction in gene expression (Graessmann et al. 1994; Hisano et al. 2003). In addition, Appanah et al. (2007) proposed that methylation of the 3′ promoter-proximal region, approximately 300 bp downstream of the TSS, may dramatically reduce transcription initiation efficiency by relating alteration of the promoter chromatin structure. The findings about the region downstream of the TSS explain why the expression levels of the *CES1A1* gene in the human kidney and liver are different. In the genome obtained from the kidney, the region downstream of the TSS was methylated compared with that obtained from the liver (Figure 6).

When the amplified region of 726 bp was segregated into three regions (I–III), there was no apparent difference between methylation in region I in the kidney and that in region I in the liver. In region II, CpGs in the kidney were slightly methylated compared with those in the liver. In region III, which is included in the CpG island of the gene, CpGs in the kidney were more methylated than those in the liver. Hence, methylation in region III plays an important role in the difference between *CES1A1* gene expression in the kidney and that in the liver, and methylation in region II may also have a minor role in that difference. It was previously suggested that methylated and inactive promoters are occupied by nucleosomes in the silenced state of the *MLH1* gene (Lin et al. 2007). Nucleosomal occupancy is involved in methyl-binding proteins (Li et al. 2007). In such a mechanism, *CES1A1* gene expression may also be repressed possibly by methylation in the promoter region, particularly at position −24 immediately upstream of the TSS. There was partial discordance of the methylation pattern between cell lines and tissues. To explain this phenomenon, we focused on three points. First, the tissues consist of several kinds of cells, whereas the cell lines consist of almost one kind of cell. The hypermethylated clones were observed in the genomes obtained from all liver tissues at almost the same rate. There is a possibility that the hypermethylated clones were obtained from hepatic non-parenchymal cells. Second, there is a possibility that a cell line is partially different from a normal tissue in DNA methylation pattern. Third, although DNA methylation pattern can change with age (Bjornsson et al. 2008), the ages of tissue specimens and cell line samples used in this study were not the same.

Since CESs catalyse the hydrolysis of PNPA, we performed enzyme assay using PNPA. As the result of treatment with 5-aza-dC to HEK293 cells, the activity of hydrolysis of PNPA was increased approximately 25% compared with that of control. But the increases of the activity were lower than expected. We thought that more time after transcription to observe the change caused by single or combined treatment with 5-aza-dC and TSA may be necessary, but after transcription how fast functional CESs are generated is unknown. It is known that the half-life of a rat liver CES isozyme is 42 h (Heymann et al. 1979). Although the half-life of CES1A1 protein is unknown, the finding of half-life for CES1A1 would be explain the difference between the activity of hydrolysis caused by treatment with 5-aza-dC to HEK293 cells and the activity of hydrolysis of HuH-7 cells.

The present study provides information on the metabolism and disposition of prodrugs associated with CES1A1. For example, CES1A1 converts oseltamivir, an inhibitor of viral neuraminidase, into the active metabolite Ro 64-0802, oseltamivir carboxylate, in the human liver (Shi et al. 2006). Oseltamivir can cross the blood–brain barrier (BBB), and its brain penetration at the BBB is limited by P-glycoprotein (P-gp) (Morimoto et al. 2008; Ose et al. 2008). The degree of penetration of Ro 64-0802 at the BBB is lower than that of oseltamivir. According to Ose et al. (2009), Ro 64-0802 in the brain is eliminated across the BBB by its active efflux by multidrug resistance-associated protein 4 (Mrp4, Abcc4) and organic anion transporter 3 (Oat3, Slc22a8) in mice, although Oat3 may not affect its brain distribution in a steady state. These findings indicate that if *CES1A1* gene expression in the human liver or brain capillary decreases, the distribution of oseltamivir to the brain may increase. It is known that oseltamivir and Ro 64-0802 affect neuronal excitability in rat hippocampal slices (Izumi et al. 2007). Recently, the relationship between abnormal behaviour of children and oseltamivir medication has been studied in detail, but the relationship remains unclear. Yang et al. (2009) revealed that expression level of the human *CES1A* gene in the liver of children is lower than that in the liver of adults and that liver microsomal samples pooled from children showed approximately 15% of the activity of the samples pooled from adults in hydrolysing oseltamivir. Hence, there is a possibility that the difference in *CES1A1* gene expression between children and adults is involved in the abnormal behaviour of children. Considering that DNA methylation pattern can change with age (Bjornsson et al. 2008), we speculated that the difference in *CES1A1* gene expression between children and adults is probably due to the difference in DNA methylation levels in the region around the TSS. This point of view may help to understand the relationship between abnormal behaviour and oseltamivir medication.

The present study revealed that DNA methylation is involved in *CES1A1* gene expression. The difference between the gene expression in the human kidney and that in the human liver may arise from the difference in DNA methylation levels in the region around the TSS.

Acknowledgements

Declaration of interest

References

Anttila S, Hakkola J, Tuominen P, Elovaara E, Husgafvel-Pursiainen K, Karjalainen A, Hirvonen A, Nurminen T. (2003). Methylation of cytochrome P4501A1 promoter in the lung is associated with tobacco smoking. Cancer Res 63(24):8623-8.

Aoki M, Terada T, Kajiwara M, Ogasawara K, Ikai I, Ogawa O, Katsura T, Inui K. (2008). Kidney-specific expression of human organic cation transporter 2 (OCT2/SLC22A2) is regulated by DNA methylation. Am J Phys 295(1):F165-70.

Appanah R, Dickerson DR, Goyal P, Groudine M, Lorincz MC. (2007). An unmethylated 3' promoter-proximal region is required for efficient transcription initiation. PLoS Genet 3(2):e27.

Bencharit S, Morton CL, Hyatt JL, Kuhn P, Danks MK, Potter PM, Redinbo MR. (2003). Crystal structure of human carboxylesterase 1 complexed with the Alzheimer's drug tacrine: from binding promiscuity to selective inhibition. Chem Biol 10(4):341-9.

Bird A. (2002). DNA methylation patterns and epigenetic memory. Genes Develop 16(1):6-21.

Bird AP, Wolffe AP. (1999). Methylation-induced repression - belts, braces, and chromatin. Cell 99(5):451-4.

Bjornsson HT, Sigurdsson MI, Fallin MD, Irizarry RA, Aspelund T, Cui H, Yu W, Rongione MA, Ekstrom TJ, Harris TB, Launer LJ, Eiriksdottir G, Leppert MF, Sapienza C, Gudnason V, Feinberg AP. (2008). Intra-individual change over time in DNA methylation with familial clustering. J Am Med Assoc 299(24):2877-83.

Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21(1):103-7.

Coffee B, Zhang F, Warren ST, Reines D. (1999). Acetylated histones are associated with FMR1 in normal but not fragile X-syndrome cells. Nat Genet 22(1):98-101.

El-Osta A, Kantharidis P, Zalcberg JR, Wolffe AP. (2002). Precipitous release of methyl-CpG binding protein 2 and histone deacetylase 1 from the methylated human multidrug resistance gene (MDR1) on activation. Molecular Cell Biol 22(6):1844-57.

Fukami T, Nakajima M, Maruichi T, Takahashi S, Takamiya M, Aoki Y, McLeod HL, Yokoi T. (2008). Structure and characterization of human carboxylesterase 1A1, 1A2, and 1A3 genes. Pharmacogenet Genomics 18(10):911-20.

Gagnon JF, Bernard O, Villeneuve L, Tetu B, Guillemette C. (2006). Irinotecan inactivation is modulated by epigenetic silencing of UGT1A1 in colon cancer. Clin Cancer Res 12(6):1850-8.

Graessmann A, Sandberg G, Guhl E, Graessmann M. (1994). Methylation of single sites within the herpes simplex virus tk coding region and the simian virus 40 T-antigen intron causes gene inactivation. Mol Cell Biol 14(3):2004-10.

Heymann E, Mentlein R, Schmalz R, Schwabe C, Wagenmann F. (1979). A method for the estimation of esterase synthesis and degradation and its application to evaluate the influence of insulin and glucagon. Eur J Biochem 102(2):509-19.

Hisano M, Ohta H, Nishimune Y, Nozaki M. (2003). Methylation of CpG dinucleotides in the open reading frame of a testicular germ cell-specific intronless gene, Tact1/Actl7b, represses its expression in somatic cells. Nucleic Acids Res 31(16):4797-804.

Hosokawa M. (2008). Structure and catalytic properties of carboxylesterase isozymes involved in metabolic activation of prodrugs. Molecules (Basel) 13(2):412-31.

Hosokawa M, Furihata T, Yaginuma Y, Yamamoto N, Watanabe N, Tsukada E, Ohhata Y, Kobayashi K, Satoh T, Chiba K. (2008). Structural organization and characterization of the regulatory element of the human carboxylesterase (CES1A1 and CES1A2) genes. Drug Metab Pharmacokinet 23(1):73-84.

Hosokawa M, Maki T, Satoh T. (1987). Multiplicity and regulation of hepatic microsomal carboxylesterases in rats. Mol Pharmacol 31(6):579-84.

Ishizuka H, Konno K, Naganuma H, Sasahara K, Kawahara Y, Niinuma K, Suzuki H, Sugiyama Y. (1997). Temocaprilat, a novel angiotensin-converting enzyme inhibitor, is excreted in bile via an ATP-dependent active transporter (cMOAT) that is deficient in Eisai hyperbilirubinemic mutant rats (EHBR). J Pharmacol Exp Ther 280(3):1304-11.

Izumi Y, Tokuda K, O'Dell K A, Zorumski CF, Narahashi T. (2007). Neuroexcitatory actions of Tamiflu and its carboxylate metabolite. Neurosci Lett 426(1):54-8.

Kikuchi R, Kusuhara H, Hattori N, Kim I, Shiota K, Gonzalez FJ, Sugiyama Y. (2007). Regulation of tissue-specific expression of the human and mouse urate transporter 1 gene by hepatocyte nuclear factor 1 alpha/beta and DNA methylation. Mol Pharmacol 72(6):1619-25.

Kondo Y, Shen L, Issa JP. (2003). Critical role of histone methylation in tumor suppressor gene silencing in colorectal cancer. Mol Cell Biol 23(1):206-15.

Kumaki Y, Oda M, Okano M. (2008). QUMA: quantification tool for methylation analysis. Nucleic Acids Res 36(Web Server issue):W170-5.

Larsen F, Gundersen G, Lopez R, Prydz H. (1992). CpG islands as gene markers in the human genome. Genomics 13(4):1095-107.

Li B, Carey M, Workman JL. (2007). The role of chromatin during transcription. Cell 128(4):707-19.

Li LC, Dahiya R. (2002). MethPrimer: designing primers for methylation PCRs. Bioinformatics 18(11):1427-31.

Lin JC, Jeong S, Liang G, Takai D, Fatemi M, Tsai YC, Egger G, Gal-Yam EN, Jones PA. (2007). Role of nucleosomal occupancy in the epigenetic silencing of the MLH1 CpG island. Cancer Cell 12(5):432-44.

McGurk KA, Brierley CH, Burchell B. (1998). Drug glucuronidation by human renal UDP-glucuronosyltransferases. Biochem Pharmacol 55(7):1005-12.

Morimoto K, Nakakariya M, Shirasaka Y, Kakinuma C, Fujita T, Tamai I, Ogihara T. (2008). Oseltamivir (Tamiflu) efflux transport at the blood-brain barrier via P-glycoprotein. Drug Metab Disposit 36(1):6-9.

Ose A, Ito M, Kusuhara H, Yamatsugu K, Kanai M, Shibasaki M, Hosokawa M, Schuetz JD, Sugiyama Y. (2009). Limited brain distribution of [3R,4R,5S]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate (Ro 64-0802), a pharmacologically active form of oseltamivir, by active efflux across the blood-brain barrier mediated by organic anion transporter 3 (Oat3/Slc22a8) and multidrug resistance-associated protein 4 (Mrp4/Abcc4). Drug Metab Disposit 37(2):315-21.

Ose A, Kusuhara H, Yamatsugu K, Kanai M, Shibasaki M, Fujita T, Yamamoto A, Sugiyama Y. (2008). P-glycoprotein restricts the penetration of oseltamivir across the blood-brain barrier. Drug Metab Disposit 36(2):427-34.

Peters AH, Mermoud JE, O'Carroll D, Pagani M, Schweizer D, Brockdorff N, Jenuwein T. (2002). Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. Nat Genet 30(1):77-80.

Razin A. (1998). CpG methylation, chromatin structure and gene silencing - a three-way connection. EMBO J 17(17):4905-8.

Sanghani SP, Quinney SK, Fredenburg TB, Davis WI, Murry DJ, Bosron WF. (2004). Hydrolysis of irinotecan and its oxidative metabolites, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin, by human carboxylesterases CES1A1, CES2, and a newly expressed carboxylesterase isoenzyme, CES3. Drug Metab Disposit 32(5):505-11.

Satoh T, Hosokawa M. (2006). Structure, function and regulation of carboxylesterases. Chemico-Biol Interact 162(3):195-211.

Satoh T, Taylor P, Bosron WF, Sanghani SP, Hosokawa M, La Du BN. (2002). Current progress on esterases: from molecular structure to function. Drug Metab Disposit 30(5):488-93.

Saxonov S, Berg P, Brutlag DL. (2006). A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci USA 103(5):1412-17.

Shi D, Yang J, Yang D, LeCluyse EL, Black C, You L, Akhlaghi F, Yan B. (2006). Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is

Review

(Special Topic)

Carboxylesterases: structure, function and polymorphism in mammals

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This review covers current developments in molecular-based studies of the structure and function of carboxylesterases. To allay the confusion of the classic classification of carboxylesterase isozymes, we propose a novel nomenclature and classification of mammalian carboxylesterases on the basis of molecular properties. Mechanisms of the regulation of the gene expression of carboxylesterases by xenobiotics, and the involvement of carboxylesterase in drug metabolism are also described. The novel biomarker for organophosphate pesticide exposure developed here is much more useful and reliable than cholinesterase inhibition. © Pesticide Science Society of Japan

Keywords: carboxylesterase, genetic polymorphism, molecular structure, classification, novel biomarker.

1. Introduction

The present review highlights the importance of structure in delineating overall function, substrate specificity, regulation and localization of carboxylesterases (CEs). Structural considerations emerge from the genes encoding the family of enzymes. Sequence homology typically yields insights into the evolutionary relationship between the members and the conserved and divergent areas of sequence. Diversity in the structure and ultimately function and cellular localization of the gene product is achieved through gene doubling and divergence, alternative mRNA processing, and post-translational modification.

The acetylcholinesterases (AChE) and CEs belong to a protein superfamily termed the α,β-hydrolase-fold family[1] in which members may have highly specialized functions, as is the case for AChE and juvenile hormone esterase. These members show a high degree of selectivity for a neurotransmitter or a hormone, respectively. Namely, other members of the family, such as butyrylcholinesterase (BuChE) or the wide variety of CEs found in tissues and plants, show greater activity in substrates with which they catalyze. Hence, they serve a protective and clearing function for foreign substrates encountered through the diet or other routes of exposure. This family of enzymes also shows great differences in the cells in which they are expressed; some are found in multiple cell types, whereas others show highly selective expression.

Finally, within the cell itself, we observed distinctive localizations; some of the enzymes are destined for export into the plasma, whereas others are associated with the cell membrane with its catalytic function directed extracellularly. Others may be retained within subcellular organelles, such as the endoplasmic reticulum (ER), whereas still others are found in the cytoplasm.

Unlike the serine superfamily, it is clear that paraoxonases are not serine or cysteine esterases, although we know less about the structure of paraoxonases. Rather, they belong to a discrete family of esterases, most likely those in which a divalent metal is required for catalysis. Considerable progress has been made recently in their purification and structural elucidation. Importantly, inroads also have been made in detecting natural substrates for paraoxonases, and lactones emerge as a prime candidate across several species.

The expression profiles of gene expression encoding esterases are highly regulated during development by nutrition

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status, hormonal factors, and xenobiotics. Although the consequences of regulating esterases by drugs and chemicals have been intensively studied, relatively little is known about the mechanisms by which esterases are regulated by physiological factors. This regulation has several potential consequences for the pharmacological and toxicological actions of drugs and chemicals in humans and animals in different developmental stages and nutritional states.

2. Mammalian CEs

Carboxylesterases (EC 3.1.1.1) are members of an α,β-hydrolase-fold family and are found in various mammalian species.[2–14] These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs and chemicals to the respective free acids. They are also involved in the detoxification or metabolic activation of various drugs, pesticides, environmental toxicants and carcinogens. Carboxylesterases catalyze the hydrolysis of endogenous compounds, such as short- and long-chain acylglycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters.[215–22] We have reviewed the characteristics of CEs in relation to the metabolism of xenobiotics.[23–26] Multiple isozymes of hepatic microsomal CE exist in various animal species[27–29] and some of these isozymes are involved in the metabolic activation of certain carcinogens, as well as being associated with hepatocarcinogenesis.[17] It has been suggested that CEs can be classified into five major groups, CES1 to CES5, according to the homology of the amino acid sequence,[23,25,26] and the majority of CEs that have been identified to belong to the CES1 or CES2 family.

Striking species differences have also been shown;[28–30] for example, Inoue et al.[31] showed that esterase activity in the dog intestine is very weak and produced no appreciable active band in disk electrophoresis coupled with the staining of esterase activity. On the other hand, esterase activities were observed in the intestines of other species (human,[25,30,31] rat,[30,31] mouse,[31] guinea pig,[31] rabbit,[31] dog[30,31] and monkey[30]) and were found to produce a few active bands in an electrophoretic assay.

It is thought that CEs are one of the major determinants of the pharmacokinetics and pharmacodynamics of ester drugs or ester prodrugs. Since the pharmacological data on ester prodrugs obtained from preclinical experiments are generally used as references for human studies, it is important to clarify the biochemical properties of each CE isozyme, including substrate specificity, tissue distribution, and transcriptional regulation.

This review addresses the significant differences in the molecular structure and function of recently identified CEs, and proposes a novel nomenclature for mammalian CE isozymes based on the nucleotide sequences of the genes encoding the individual isozymes. In addition, the different structure-activity relationships of substrates with each CE family and the genetic polymorphism of CE genes are also described.

3. Novel Classification and Nomenclature of Mammalian CEs

According to the classification of esterase by Aldridge,[33] the serine superfamily of esterase, i.e., AChE, BuChE and CE, falls into the B-esterase group. CE isozymes were initially classified by their substrate specificities and isoelectric point; however, this classification is ambiguous in overlapping substrate specificities. A single esterolytic reaction is frequently mediated by several kinds of enzyme. Recent studies on esterases, as on other enzymes concerned with xenobiotic metabolism, have afforded evidence of multiple forms.

It seems almost impossible to classify these CE isozymes based on their substrate specificity along the lines of the International Union of Biochemistry (I.U.B.) classification, because the individual hydrolases exhibit properties of CE, lipase or both. Mentlein et al.[16] proposed classifying these hydrolases as "unidentified CEs" (EC 3.1.99.1 to 3.1.99.x). Based on the high homology and similarity of the amino acid sequence alignment of the encoding genes, we tried to classify CE isozymes into five families: CES 1, CES 2, CES 3, CES4 and CES 5[24,25] (Fig. 1).

The CES 1 family includes the major form of CE isozymes (more than 60% homology with human CES). Thus, they could be divided into eight subfamilies: CES1A, CES1B, CES1C, CES1D, CES1E, CES1F, CES1G, CES1H. Most of the CES1 families, except CES1G, are mainly expressed in the liver. The CES 1A subfamily includes the major forms of human CEs,[18,19,34,36–38] and the major isoforms of rat,[9] dog,[19] rabbit[37] and mouse[35] CE. The CES1B subfamily includes the major isoforms of rat,[9] mouse[20] and hamster[47] CE, and CES1C includes the major isoforms of dog,[18,19] cat[50] and human[38] CE. The CES 1H subfamily includes RL1 (CES1H4), mouse ES 4 (CES1H1) and hydrolase B (CES1H3) and C (CES1H2), which catalyze long-chain acyl-CoA hydrolysis.[6,9,18,27,39] Members of the CES 1G family are not retained in ER, which are secreted into the blood from the liver,[8,35] and these families are all secretary-type CEs. It is interesting that the CES1G family is found in only rats and mice, but not humans, and they are all secretary types of CESs. Although a high level of CES1 activity is detected in the blood of rats and mice, no activity is detected in human blood.

In contrast, the CES2 family is mainly expressed in the small intestine. It includes human intestinal CE (CES2A1),[34,40–44] rat CES2 (CES2A10),[22] rat intestinal CE RL4 (rCES2) (CES2A6),[45] rabbit form 2[46] and hamster AT51 (CES2A11).[47] CES3 includes ES-male (CES3A2) and human CES3 (CES3A1).[48,49] Human CES3 (CES3A1) has about 40% amino acid sequence identity with both CEA1A1 and CES2A1, and is expressed in the liver and gastrointestinal tract at an extremely low level in comparison with CES1A1 and CES2A1.[49]

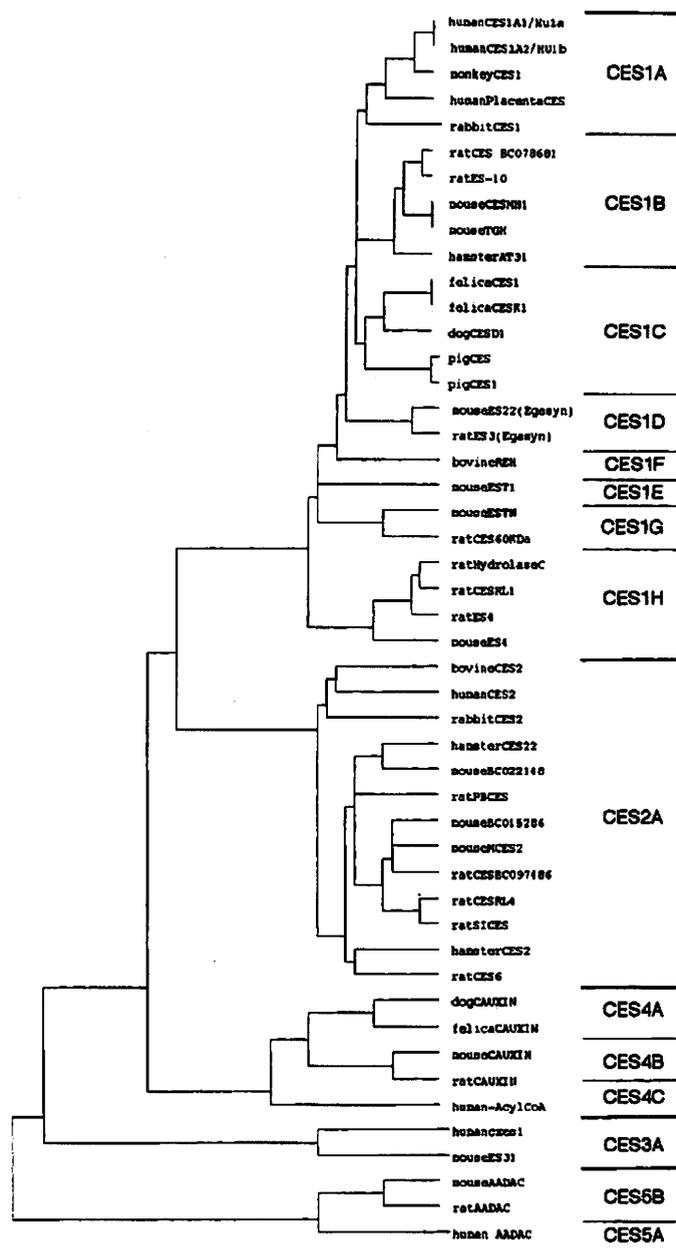The CES4 family includes CE-like urinary excreted protein

Fig. 1. Phylogenic tree and nomenclature of CE families. Carboxylesterase isozymes are classified into five families, CES1, CES2, CES3, CES4 and CES5. Each family is also divided into subfamilies.

(CAUXIN) (CES4A2), which is excreted as a major urinary protein in cat urine.[50] The CES 5 family includes 46.5-kDa CE isozymes,[51] which have a different structure from the structures of isozymes in other CE families. Esterase (ES) 46.5-kDa from mouse liver[52] and amide hydrolase from monkey liver[10] probably belong to this family. These groupings are similar to the results of phylogenic analysis (Fig. 1).

4. Structure and Catalytic Mechanism of CE Isozymes

It has been shown that several proteins in the ER lumen have a common carboxy-terminal sequence, KDEL-COOH, and

that the structural motif is essential for retention of the protein on the luminal side of the ER through the KDEL receptor bound to the ER membrane.[53-55] Ozols[46] and Korza and Ozols[56] established the primary structures of two microsomal esterases purified from rabbit liver and designated them 60-kDa esterase forms 1 and form 2, respectively. These two forms of CE have a consensus sequence for the ER retention tetra-peptide (HTEL or HIEL in the one-letter code) that is recognized on the luminal side of the KDEL receptor. The HXEL-COOH motif is also essential for retention of the protein on the luminal side of the ER through the KDEL receptor bound to ER membrane.[53-55] Robbi et al.[57] reported cDNA cloning of rat liver CES1B4 (ES-10), and that was the first report to show that cDNA of liver CarbE has the consensus sequence of the ER retention tetrapeptide (HVEL-COOH).

Later, Robbi and Beaufay[58] isolated a cDNA clone of another rat liver CES1D2 (ES-3, egasyn), which encoded the consensus sequence of the ER retention tetrapeptide (HTEL-COOH). The other clone, encoded egasyn, is an accessory protein of β-glucuronidase in liver microsomes.[59] Egasyn is identical to CE, and it binds β-glucuronidase via its CE active site. In rats and mice, the carboxyl terminal amino acid sequence of clone rat CES-60KDa (CES1G1) and mouse

Es-N (CES1G2) is HTEHK-COOH, which can not bind to KDEL receptor, and these isozymes are secreted into blood.[35]

Carboxylesterases have a signal peptide of 17 to 22 amino acid residues near the N-terminal, including hydrophobic amino acid. In the CES1 family, exon 1 encodes a signal peptide.[60,61] In the CES1 family, a bulky aromatic residue (Trp) followed by a small neutral residue (Gly) directly precede the cleavage site.[62] Carboxylesterases have four Cys residues that may be involved in specific disulfide bonds. Among them, Cys98 is the most highly conserved residue in many CE isozymes. Cygler et al.[1] reported the important alignment of a collection of related amino acid sequences of esterase, lipase and related proteins based on X-ray structures of Torpedo californicaAChE and Geotrichum candidum lipase.

According to the literature, Ser203, Glu336 and His450 form a catalytic triad, and Gly124-Gly125 may be part of an oxyanion hole (Fig. 2). These residues are also highly conserved among CE isozymes. Site-specific mutation of Ser203 to Thr203, Glu336 to Ala336, or His450 to Ala 450 greatly reduced the CE activity towards substrates; therefore, this mutagenesis confirmed the role of Glu336 and His450 in forming a putative charge relay system with active site Ser203.[24]

Frey et al.[63] reported that the formation of low barrier hy-
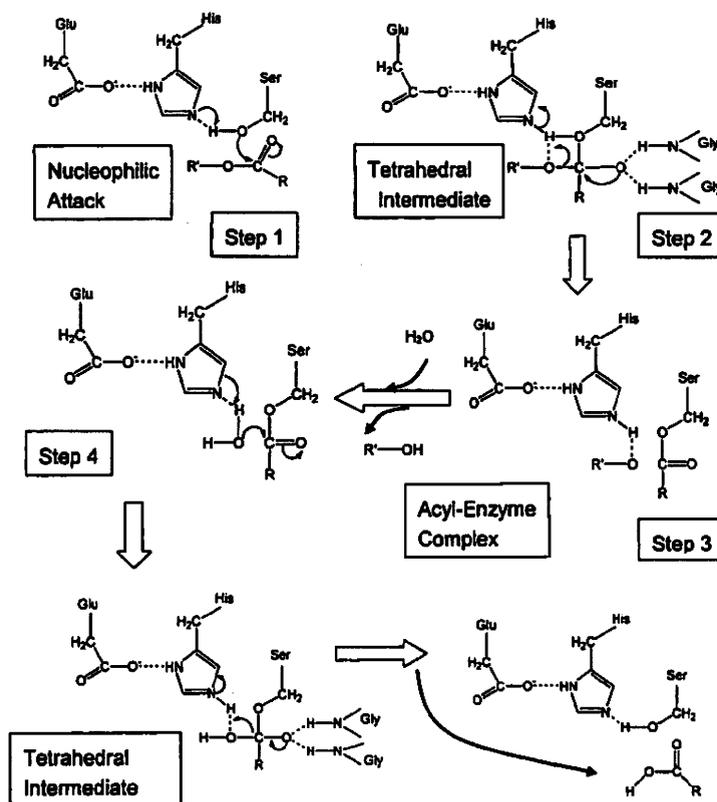

Fig. 2. Proposed mechanism of action of CE. Conformation of the Ser-His-Glu catalytic triad in CE.

drogen bonds between His and Asp (Glu for CE) facilitates nucleophilic attack by the β-OH group of Ser on the acyl carbonyl group of peptide in chymotrypsin. The catalytic triad in the tetrahedral addition intermediate is stabilized by low barrier hydrogen bonds. According to their theory, we speculated that the low barrier hydrogen bond between Glu336 and His 450 facilitates nucleophilic attack by the β-OH group of Ser203 on the carbonyl group of the substrate in CE (Fig. 2).

The mechanism of CE could thus be divided into the following steps: 1) The enzyme substrate complex form, positioning the substrate in the correct orientation for reaction. 2) Hydrolysis of the ester bond starts with an attack by the oxygen atom of the hydroxy group of Ser203 on the carbonyl carbon atom of the ester bond. 3) The hydrogen bonds between the negatively charged oxygen of the tetrahedral intermediate and the N–H group of Gly123 and Gly124 stabilize the negatively charged oxygen (O^-). This configuration, in which negatively charged carboxyl oxygen is hydrogen bonded to two N–H groups, is called an oxyanion hole.

In the general acid-catalyzed step, the ester bond breaks, and the leaving group picks up a proton from the imidazolium ion of His$_{450}$. The acyl portion of the original ester bond remains bound to the enzyme as an acyl-enzyme intermediate. The alcohol component (R'-OH) diffuses away, completing the acylation stage of the hydrolytic reaction. 4) A water molecule attacks the acyl-enzyme intermediate to give a second *tetrahedral intermediate.* 5) His$_{450}$ then donates the proton to the oxygen atom of Ser203, which then releases the acid component of the substrate. The acid component diffuses away and the enzyme is ready for catalysis.

The tetrahedral transition state is stabilized by the formation of low barrier hydrogen bonds between His450 and Glu336. This low barrier hydrogen bond-facilitated mechanism includes weak hydrogen bonds between the oxyanion (O^-) and peptide N–H bonds contributed by Gly123 and Gly124, which stabilize the tetrahedral adduct on the substrate side of the transition state (Fig. 2). Formation of the acyl-enzyme complex in the next step requires removal of a proton from His$_{450}$, so that the tetrahedral intermediate is disrupted in the acyl-enzyme intermediate. When the unbound portion of the alcohol group of the first product of the substrate has diffused away, a second step which the deacylation step is essentially the reverse of the acylation step occurs, with a water molecule substituting for the alcohol group of the original substrate.

It is of interest that the sequences required for the hydrolytic capability at the catalytic triad (Glu, His, Ser) of CE, AChE, BuChE, and cholesterol esterase are highly conserved. This is a common structure of α,β-hydrolase-fold families, which are responsible for the hydrolysis of endogenous and exogenous compounds. Furthermore, these elements are strongly conserved among orthologous CEs of the mouse, rat, rabbit, monkey and human.

A three-dimensional model of human CE has been pro-posed on the basis of crystal structure coordinates of AChE and overlapping active sites with pancreatic lipase and CE.[64] The modeled structure shares the overall folding and topology of the proteins identified in the recently published crystal structures of the rabbit[65] and human CE.[66,67] Carboxylesterase has a three-dimensional α,β-hydrolase-fold structure, which is a structural feature of all lipases.[66] In general, the structure of CE may be viewed as a central catalytic domain surrounded by α,β and regulatory domains.[65,67,68] In essence, the α,β-hydrolase-fold consists of a central β-sheet surrounded by a variable number of β-helices and accommodates a catalytic triad composed of Ser, His and a carboxylic acid. This suggests that the catalytic function of these proteins is conserved across species.

The catalytic triad is located at the bottom from about a 25 Å deep active site, approximately in the center of the molecule, and is composed of a large flexible pocket on one side of Ser203 and a small rigid pocket on the opposite side.[67] The orientation and location of the active site provide an ideal hydrophobic environment for the hydrolysis of a wide variety of hydrophobic substrates.[67] The small rigid active site pocket is adjacent to the oxyanion hole formed by Gly123-124 and is lined by several hydrophobic residues.[67]

Short acyl chains would be easily accommodated within the small rigid pocket. The larger flexible active site pocket is lined by several non-polar residues and could accommodate larger or polycyclic molecules, such as cholesterol. The large pocket is adjacent to a side door secondary pore that would permit small molecules (substrates and reaction products) to enter and exit the active site.[67] Longer acyl chains may be oriented for catalysis in such a way that they extend through the side door. Indeed, the presence of a hydrophobic residue at position 423 in mouse CES1B2 and 425 in human CES1A1 is necessary for efficient hydrolysis of hydrophobic substrates, as a mutation of Met 423 of the related rat lung CE (CES1B4) to Ile increased CE activity towards a more hydrophobic substrate without affecting activity towards short-chain esters.[69]

Most CE isozymes are glycoproteins, and the carbohydrate chain is required for the enzyme activity of CEs.[3,24,26,29,34,68] Human CES2A1 contains a glycosylation site at two different positions (Asn103 and Asn267), while CES1A1 contains only one glycosylation site at Asn79. This glycosylation site is modified by a carbohydrate chain with first N-acetylglucosamine and terminal sialic acid and appears to be involved in the stabilization of the CES1A1 trimer by packing into the adjacent monomer in its crystal structure.[67]

According to the X-ray crystal structure of human CES1, this residue lines the flexible pocket adjacent to the side door.[67] Given the wide range of substrates that CEs are known to hydrolyze, the large flexible pocket confers the ability to hydrolyze many structurally distinct compounds, whereas the rigid pocket is much more selective with regard to the substrates that may be accommodated.

5. Gene Structure and Regulation of CE Isozymes

Both the murine[26] and human[13,61] CES1 genes span about 30 kb and contain 14 small exons. Recently, sequencing of the mouse and human genomes has been completed, enabling detailed sequence comparisons. Previously published sequences of individual exons, splice junctions, size of the introns and restriction sites within the murine and human CE genes are consistent with their respective genes sequenced by the mouse and human genome projects. Therefore, the organization of the CE gene is evolutionarily conserved in mice and humans. Previous studies have mapped the human CE gene to chromosome 16 at 16q13–q22.1.[3,36] This region is syntenic to a region of mouse chromosome 8 at 8C5. The murine CE Es22[60] and Es-N[35] have been previously mapped to chromosome 8. The completion of the mouse genome sequencing project unambiguously demonstrated that the murine CE gene was located on the minus strand of chromosome 8 at 8C5 in a cluster of six CE genes that span 260.6 kb. These six CE genes are presumed to have originated from repeated gene duplications of a common ancestral gene that encoded a CE,[61] and subsequent evolutionary divergence may occur.

Recently, we have identified a mouse liver microsomal acylcarnitine hydrolase, mCES2, as a member of the CES2 family.[45] It has been revealed that this enzyme is significantly induced by di(2-ethylhexyl)phthalate (DEHP) and shows medium- and long-chain acylcarnitine hydrolase activity[45] In addition, we have found that mCES2 is expressed in various tissues with higher levels of expression in the liver, kidney and small intestine. Subsequently, it was shown that three transcription factors, specificity protein (Sp) 1, Sp3 and upstream stimulatory factor 1, could bind to the promoter region of the mCES2 gene, leading to synergistic transactivation of the promoter.[21] Although this mechanism may explain the ubiquitous tissue expression profiles of mCES2, it is unlikely to contribute to the higher levels of mCES2 expression in the liver, kidney and small intestine; therefore, it is thought that another mechanism controls this tissue-specific transcription of the mCES2 gene.[21]

More recently, we have shown that hepatocyte nuclear factor-4 alpha (HNF-4α) can strongly enhance mCES2 gene transcription and that the involvement of HNF-4α accounts for the high expression level of mCES2 in the liver.[71] These findings are notable when the physiological roles of mCES2 are studied, since HNF-4α is involved in various hepatic functions, such as glucose, cholesterol and drug metabolism. In addition, we found that bile acid can repress mCES2 gene transcription by repressing HNF-4α-mediated transactivation.[71]

In 2008, we isolated and characterized two genes encoding the human CES1A1 (AB119997) and CES1A2 (AB119998), and cloned and sequenced the 5' flanking region of each gene in order to elucidate the structure of the promoter[72] (Fig. 3).
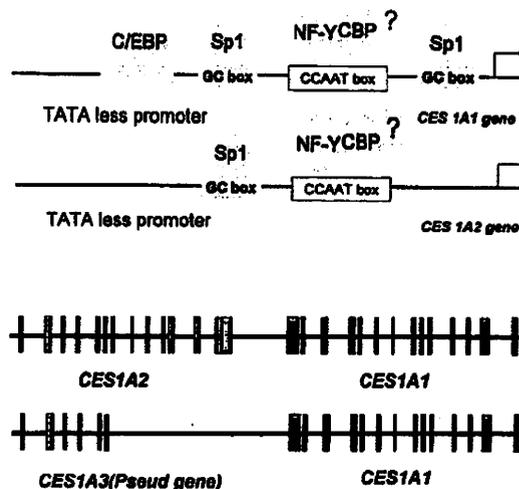
It is noteworthy that both the CES1A1 and CES1A2 genes

Fig. 3. Structure of the 5' flanking region of CES1A1 and CES1A2 genes. Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but Sp1 and C/EBP could not bind to the 5' flanking region of the CES1A2 promoter. NF-Y, nuclear factor Y; CBF, CCAAT-binding factor

are located on chromosome 16q13–q22 with a tail-to-tail structure. Comparison of the nucleotide sequences of CES1A1 and CES 1A2 genes revealed about 98% homology in 30 Kbp. There are only six nucleotide differences, resulting in four amino acid differences in the open reading frame, and all of the differences exist in exon 1.

Gene duplication has generally been viewed as a necessary source of material for the origin of evolutionary novelties, and duplicate genes evolve new functions. The majority of gene duplicates are silenced within a few million years, with the small number of survivors subsequently being subjected to strong purifying selection. Although duplicate genes may only rarely evolve new functions, the stochastic silencing of such genes may play a significant role in the passive origin of new species. Since exon 1 of the CES1 gene encodes a signal peptide region, intracellular localization of the CES1 gene product was preliminarily investigated using a signal peptide/EYFP-ER chimera protein-expressing system. It was interesting that the CES1A1 signal peptide/EYFP-ER chimera protein was localized to the endoplasmic reticulum, whereas the CES1A2 signal peptide/EYFP-ER chimera protein was distributed in the ER and cytosol.

On the other hand, CES1A2 mRNA was found to be expressed only in the human adult liver, although CES1A1 is expressed in both the human adult and fetal liver.[72] These results suggested that CES1A1 and CES1A2 have different intracellular localizations and different expression profiles in liver differentiation. We therefore investigated the transcriptional regulation of these two CE genes. Reporter gene assays and electrophoretic mobility shift assays demonstrated that

Table 1. Tissue-specific expression profile of CES1 and CES2 isozymes in mammals and humans.

Species	Isozyme	Liver	Small intestine	Kidney	Lung
Mouse	CES1	+++	−	+++	+++
	CES2	+++	+++	+++	−
Rat	CES1	+++	−	+++	+++
	CES2	−	+++	−	−
Hamster	CES1	+++	−	+++	NT
	CES2	+++	+++	−	NT
Guinea Pig	CES1	+++	+++	++	NT
	CES2	−	+	−	NT
Beagle	CES1	+++	−	NT	+++
	CES2	++	−	NT	+
Monkey	CES1	+++	++	−	NT
	CES2	+	+++	+	NT
Human	CES1	+++	−	+	+++
	CES2	+	+++	+++	−

−, undetectable, +, weakly expressed, ++, moderately expressed, +++, strongly expressed, NT, not tested.

Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but that Sp1 and C/EBP could not bind to the responsive element of the CES1A2 promoter (Fig. 3).

More recently Fukami *et al.*[73] reported that the sequences of downstream and upstream of the intron of the CES1A2 gene are identical to those of CES1A1 and CES1A3 genes, respectively. A CES1A1 variant, in which exon 1 is converted to that of the CES1A3 gene (transcript is CES1A2), has recently been identified. It was found that the CES1A2 gene is a variant of the CES1A3 pseudogene (Fig. 3). The expression level of CES1A1 mRNA is much higher than that of CES1A2 mRNA in the liver.[72] Since CES1A1 is highly variable in the individual liver,[74] it was thought that these results provided information on the individual variation of human CES1.

As shown in Table 1, human CES1 and CES2 were highly expressed in the liver and lung, and the small intestine and kidney, respectively. Knowledge of these substrate structure-activity relationships and the tissue distribution of CE isozymes is critical for predicting the pharmacokinetics and pharmacodynamics of pesticides.

6. Possible Role of CE Isozymes in Drug Metabolism

Drug-metabolizing enzymes that are present predominantly in the liver are involved in the biotransformation of both endogenous and exogenous compounds to polar products to facilitate their elimination. These reactions are categorized into phase 1 and phase 2 reactions. CE show ubiquitous tissue expression profiles with the highest levels of CE activity present in liver

microsomes in many mammals.[15-17,20,52,74,75-79] CEs are categorized as phase 1 drug-metabolizing enzymes that can hydrolyze a variety of ester-containing drugs and prodrugs. These include angiotensin-converting enzyme (ACE) inhibitors (temocapril, cilazapril, quinapril, and imidapril),[20,38,80,81] anti-tumor drugs (CPT-11 and capecitabin),[37,41,49,82-86] and narcotics (cocaine, heroin and meperidine).[11,87,88] Thus, CEs are one of the most important enzymes involved in prodrug activation, notably with respect to tissue distribution, up-regulation in tumor cells and turnover rates.

We have shown that there are some differences between these families in terms of substrate specificity, tissue distribution, immunological properties, and gene regulation.[27] Analysis of substrate structure versus catalytic efficiency for the ester or carbamate substrates has revealed that a different family of CEs recognizes different structural features of the substrate. For example, the preferential substrates for CES1A1, a human CES1 family isozyme, are thought to be compounds esterified by small alcohols, while those for CES2A1, a human CES2 family isozyme, are thought to be compounds esterified by relatively large alcohols. CES1A1, but not CES2A1, hydrolyzed the methyl ester of cocaine and the ethyl esters of temocapril, meperidine, imidapril and oseltamivir.[20,38,80,89-91]

It was interesting that procainamide inhibited CES1-mediated imidapril hydrolysis.[92] Procainamide is also known as a choline-binding pocket-specific inhibitor[93] and has been reported to competitively inhibit human BuChE.[94] Takai *et al.*[80] reported that a local anesthetic, procaine, and the anticholinergic drug oxybutynin with large alcohol substitutes are substrates for CES2 but not CES1. Procainamide is also a good substrate for CES2. Because the amino acid sequences at the active site were highly conserved among CES1, CES2 and BuChE,[23] it is reasonable to assume that procainamide inhibits CES1- mediated imidapril hydrolysis.

In contrast to the specificity of CES1 for the methyl ester of cocaine, only CES2 hydrolyzed the benzoyl ester of cocaine.[95] The benzoyl esters of cocaine, heroin and CPT-11 (irinotecan) bearing a small acyl moiety and a bulky alcohol group are good substrates for the CES2 isozyme. Irinotecan is one of the most useful anti-tumor drugs. It was interesting that BuChE hydrolyzed the benzoyl ester of cocaine, and also hydrolyzed CPT-11, but not AChE.[96-98] CPT-11 is a relatively potent and selective inhibitor of human AChE that has properties of the acute cholinergic toxicity observed in some patients.[99]

It has been suggested that although these two CE families exhibit broad substrate specificity for ester, carbamate, or amide hydrolysis, these CE isozymes exhibit distinct catalytic efficiencies that correlate with the relative size of the substrate substituents versus that of the enzyme active sites. Tissue-specific expression of CES1 and CES2 was examined by Northern blots, RT-PCR and real-time PCR analysis.

7. Genetic Polymorphism

Geshi et al.[81] first reported that a single nucleotide polymorphism (SNP), −816A/C, of the CES1A2 gene is associated with the responsiveness to an angiotensin-converting enzyme (ACE) inhibitor, imidapril, whose activity is achieved by CES1 isozyme. Recently, we re-sequenced the CES1A2 promoter region (~1 kB) in 100 Japanese hypertensive patients. Altogether, ten SNPs and one insertion/deletion (I/D) were identified, among which six SNPs and one I/D residing between −47 and −32 were in almost complete linkage disequilibrium (D'=1.00, r2=0.97).[100] They consisted of a minor and a major haplotype, the allele frequencies of which were 22% and 74%, respectively. The minor haplotype possessed two putative Sp1 binding sites while the major haplotype did not have any Sp1 binding site. The minor haplotype had higher transcription and Sp1 binding activities than the major haplotype in vitro.

Later, we studied the relationship between CES1A1 polymorphisms and CES activity in 45 human liver tissues. Namely, six single nucleotide polymorphisms (SNPs), −75G/T, −46A/G, −39A/G, −21C/G, −20G/A, −2G/C and one insertion/deletion (I/D), +71A/del were identified in the promoter region of the CES1A1 gene. The +71 A/del was significantly associated with the conversion efficacy of CPT-11 to SN38 and the level of immunoreactive CES1 protein in the liver microsomes. The +71 A/del was not associated with the CES1A1 mRNA level in the liver, and an in vitro reporter assay indicated that +71 A/del does not affect transcription. These results suggest that CES1A1+71 A/del may account, at least in part, for the individual differences in CE activity in human liver microsomes. This polymorphism of CE genes may be a good candidate for studying the pharmacogenetics of the detoxification of drugs and chemicals, including pesticides

8. Novel Biomarker of Organophosphate Exposure

The development of a sensitive biomarker which can detect pesticide poisoning at any stage is very important. In 1981, Kikuchi et al.[101] reported that rat plasma β-glucuronidase (BG) activity was increased 2 h post-treatment with organophosphorous pesticides (OPs). Subsequently, it was reported that a complex of BG and egasyn, which is an accessory protein of BG, exists in the liver microsomal membrane. Egasyn was found to be a CEs isozyme.[24,59] BG is loosely bound to egasyn, and the complex is easily dissociated by OP exposure. Subsequently, several studies reported that intake of OP by the liver causes the release of BG into plasma.[102] Fujikawa et al.[103] reported that a single administration of Ops, including O-ethyl O-4-nitrophenyl phenylphosphonothioate (EPN), acephate and chlorpyrifos and bis(p-nitrophenyl)phosphate (BNPP) as a non-OP to rats led to a 100-fold increase in plasma BG activity over the control. In human studies, Inayat-Hussain et al.[104] reported that plasma BG activity in a group

of chronically OP-exposed farmers was significantly increased compared to that in non-OP-exposed controls. In these cases, no significant difference in BuChE activity was detected between the farmers and control groups. Recently, Soltaninejad et al.[105] observed a significant increase in blood BG activity in patients severely intoxicated by OP exposure compared to controls. In 2010, Ueyama et al.[106] reported that 42 male adults were classified into two groups; the first group consisted of 21 pesticide control operators (PCOs) who had not sprayed OPs within 3 days prior to their health check (PCO1), while the second group was composed of 21 PCOs who had sprayed OP insecticides within 3 days prior to their health check (PCO2). According to the monitoring data, plasma BG activity in the PCO2 group was higher than in PCO1. In these cases, no significant decrease in BuChE was observed.

These findings suggested that blood BG can be a more sensitive biomarker of OP exposure than the inhibition of AChE and BuChE activities in humans. We concluded that the cross-sectional studies in this paper are useful for monitoring OP exposure in a population of pest control operators

9. Conclusions and Future Directions

Multiple CEs play an important role in the hydrolytic biotransformation of a vast number of structurally diverse drugs. These enzymes are major determinants of the pharmacokinetic behavior of most therapeutic agents containing an ester or amide bond. Several factors influence CE activity, either directly or at the level of enzyme regulation. In the clinical field, drug elimination is decreased and the incidence of drug-drug interactions increases when two or more drugs compete for hydrolysis by the same CE isozyme.

Exposure to chemicals or lipophilic drugs can result in the induction of CE activity. Several drug-metabolizing enzymes, such as cytochrome P450, UDPGT-glucuronosyltransferase and sulfotransferase have been extensively studied to clarify substrate specificity using molecular cloning and cell expression systems. Consequently, the novel findings obtained reveal that the substrate specificity of CE is, at least in part, explained by differences in the nucleotide sequences of the individual CE isozymes.

It is clear that membrane-bound-type CE isozymes in microsomes are required to possess the KDEL tetrapeptide motif at the carboxy terminal of the molecule. Mammalian CEs have been found to have acyl glycerol, acyl-CoA, and acyl-carnitine hydrolyzing activities in vitro; however, the physiological roles of CE remain unclear. To clarify the substrate specificity of each CE isozyme, we have begun to search for the substrate recognition site of each isozyme.

In the present review, we described the substrate specificity and tissue-specific expression profile of CE isozymes; therefore, the successful design of ester-containing drugs will be greatly improved by further detailed analysis of the mechanism of action and substrate recognition sites of CE isozymes

in mammals.

In conclusion, the molecular-based information on CEs in this review is useful to understand the multiplicity and substrate specificity of the CE family associated with the efficacy, side effects and toxicity of chemicals.

References

1) M. Cygler, J. D. Schrag, J. L. Sussman, M. Harel, I. Silman, M. K. Gentry and B. P. Doctor BP: *Protein Sci.* **2**, 366–382 (1993).

2) M. Hosokawa: *Xenobiotic Metab. Dispos.* **5**, 185–195 (1990).

3) D. L. Kroetz, O. W. McBride and F. J. Gonzalez: *Biochemistry* **32**, 11606–11617 (1993).

4) M. R. Brzezinski, T. L. Abraham, C. L. Stone, R. A. Dean and W. F. Bosron: *Biochem. Pharmacol.* **48**, 1747–1755 (1994).

5) E. W. Morgan, B. Yan, D. Greenway, D. R. Petersen and A. Parkinson: *Arch. Biochem. Biophys.* **315**, 495–512 (1994).

6) B. Yan, D. Yang, M. Brady and A. Parkinson: *J. Biol. Chem.* **269**, 29688–29696 (1994).

7) B. Yan, D. Yang, M. Brady and A. Parkinson: *Arch. Biochem. Biophys.* **316**, 899–908 (1995).

8) B. Yan, D. Yang, P. Bullock and A. Parkinson: *J. Biol. Chem.* **270**, 19128–19134 (1995).

9) B. Yan, D. Yang and A. Parkinson: (1995). *Arch. Biochem. Biophys.* **317**, 222–234 (1995).

10) K. Kusano, T. Seko, S. Tanaka, Y. Shikata, T. Ando, S. Ida, M. Hosokawa, T. Satoh, T. Yuzuriha and T. Horie: *Drug. Metab. Didpos.* **24**, 1186–1191(1996).

11) M. R. Brzezinski, B. J. Spink, R. A. Dean, C. E. Berkman, J. R. Cashman and W. F. Bosron: *Drug Metab. Dispos.* **25**, 1089–1096 (1997).

12) T. Langmann, C. Aslanidis, M. Schuierer and G. Schmitz: *Biochem. Biophys. Res. Commun.* **230**, 215–219 (1997).

13) T. Langmann, A. Becker, C. Aslanidis, F. Notka, H. Ullrich, H. Schwer and G. Schmitz: *Biochem. Biophys. Acta* **1350**, 65–74(1997).

14) P. Ellinghaus, U. Seedorf and G. Assmann: *Biochim. Biophys. Acta* **1397**, 175–179 (1998).

15) R. Mentlein, S. Heiland and E. Heymann: *Arch. Biochem. Biophys.* **200**, 547–559 (1980).

16) R. Mentlein and E. Heymann: *Biochem. Pharmacol.* **33**, 1243–1248 (1984).

17) T. Maki, M. Hosokawa, T. Satoh and K. Sato: *Jpn. J. Cancer Res.* **88**, 800–806 (1991).

18) M. Hosokawa and T. Satoh: *7th North American ISSX Meeting*, San Diego (1996).

19) M. Hosokawa, K. Suzuki, D. Takahashi, M. Mori, T. Satoh and K. Chiba: *Arch. Biochem. Biophys.* **389**, 245–253 (2001).

20) T. Furihata, M. Hosokawa, N. Koyano, T. Nakamura, T. Satoh and K. Chiba: *Drug Metab. Dispos.* **32**, 1170–1177 (2004).

21) T. Furihata, M. Hosokawa, T. Satoh and K. Chiba: *Biochem. J.* **384**, 101–110 (2004).

22) T. Furihata, M. Hosokawa, A. Fujii, M. Derbel, T. Satoh and K. Chiba: *Biochem. Pharmacol.* **69**, 1287–1297 (2005).

23) T. Satoh and M. Hosokawa: *Toxicol. Lett.* **82**, 439–445 (1995).

24) T. Satoh and M. Hosokawa: *Annu. Rev. Pharmacol. Toxicol.* **38**, 257–288 (1998).

25) T. Satoh and M. Hosokawa: *Chem. Biol. Interact.* **162**, 195–211 (2006).

26) M. Hosokawa, T. Furihata, Y. Yaginuma, N. Yamamoto, N. Koyano, A. Fujii, Y. Nagahara, T. Satoh and K. Chiba: *Drug Metab. Rev.* **39**, 1–15 (2007).

27) M. Hosokawa, T. Maki and T. Satoh: *Mol. Pharmacol.* **31**, 579–584 (1987).

28) M. Hosokawa, K. Hirata, F. Nakata, T. Suga and T. Satoh: *Drug Metab. Dispos.* **22**, 889–894 (1994).

29) M. Hosokawa, T. Maki and T. Satoh: *Arch Biochem. Biophys.* **277**, 219–227 (1990)

30) T. Prueksaritanont, L.M. Gorham, J. H. Hochman, L. O. Tran and K. P. Vyas: *Drug Metab. Dispos.* **24**, 634–642 (1996).

31) M. Inoue, M. Morikawa, M. Tsuboi and M. Sugiura: *Jpn. J. Pharmacol.* **29**, 9–16 (1979).

32) C. M. Mansbach, and P. Nevin: *J. Lipid Res.* **39**, 963–968 (1998).

33) W. N. Aldridge WN: *Chem. Biol. Interact.* **87**, 5–13 (1993).

34) T. Imai, M. Taketani, M. Shii, M. Hosokawa and K. Chiba: *Drug Metab. Dispos.* **34**, 1734–1741 (2006).

35) M. Ovnic, K. Tepperman, S. Medda, R. W. Elliott, D. A. Stephenson, S. G. Grant and R. E. Ganschow: *Genomics* **9**, 344–354 (1991).

36) F. Zschunke, A. Salmassi, H. Kreipe, F. Buck, M. R. Parwaresch and H. J. Radzun: *Blood* **78**, 506–512 (1991).

37) P. M. Potter, C. A. Pawlik, C. L. Morton, C. W. Naeve and M. K. Danks: *Cancer Res.* **58**, 2646–2651 (1998).

38) M. Mori, M. Hosokawa, Y. Ogasawara, E. Tsukada and K. Chiba: *FEBS Lett.* **458**, 17–22 (1999).

39) M. Robbi, R. Van Schaftingen and H. Beaufay: *Biochem. J.* **313**, 821–826 (1996).

40) H. Schwer, T. Langmann, R. Daig, A. Becker, C. Aslanidis and G. Schmitz: *Biochem. Biophys. Res. Commun.* **233**, 117–120 (1997).

41) R. Humerickhouse, K. Lohrbach, L. Li, W. F. Bosron and M. E. Dolan: *Cancer Res.* **60**, 1189–1192 (2000).

42) M. Taketani, M. Shii, K. Ohura, S. Ninomiya and T. Imai: *Life Sci.* **81**, 924–932 (2007).

43) J. Yang, D. Shi, D. Yang, X. Song and B. Yan: *Mol. Pharmacol.* **72**, 686–694 (2007).

44) D. Shi, J. Yang, D. Yang and B. Yan: *Toxicology* **254**, 97–105 (2008).

45) T. Furihata, M. Hosokawa, F. Nakata, T. Satoh and K. Chiba: *Arch. Biochem. Biophys.* **416**, 101–109(2003).

46) J. Ozols: *J. Biol. Chem.* **264**, 12533–12545 (1989).

47) T. Sone, M. Isobe, E. Takabatake and C. Y. Wang: *Biochim. Biophys. Acta* **1207**, 138–142 (1994).

48) K. Aida, R. Moore and M. Negishi: *Biochim. Biophys. Acta* **1174**, 72–74 (1993).

49) S. P. Sanghani, S. K. Quinney, T. B. Fredenburg, W. I. Davis, D. J. Murry and W .F. Bosron: *Drug Metab. Dispos.* **32**, 505–511 (2004).

50) M. Miyazaki, T. Yamashita, M. Hosokawa, H. Taira and A. Suzuki: *Comp. Biochem. Physiol. Biochem. Mol. Biol.* **145**, 270–277 (2006).

51) M. R. Probst, P. Jeno and U. A. Meyer: *Biochem. Biophys. Res.*