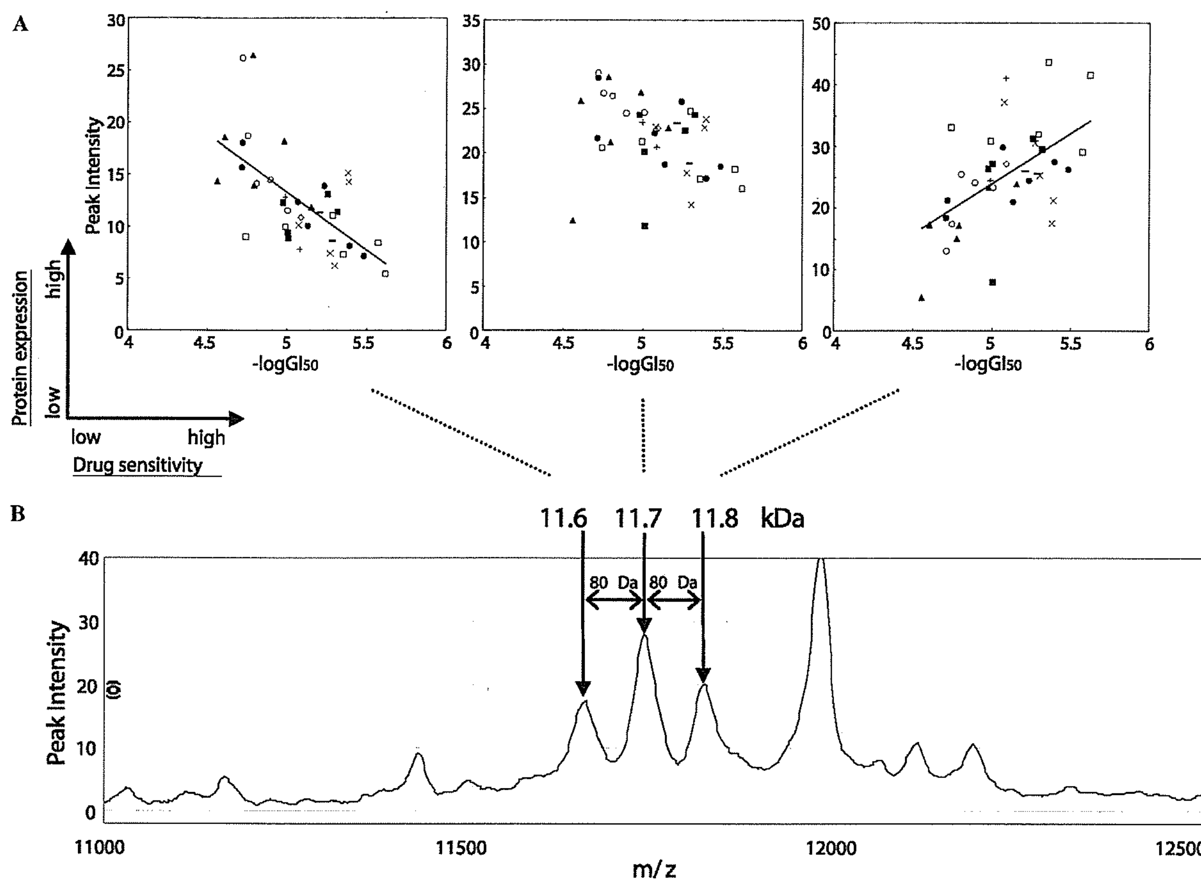

Fig. 1. Selection of target biomarker candidates determining chemosensitivity. (A) Relationship between the expression levels of the three molecules, 11.6 (left), 11.7 (middle), and 11.8 (right) kDa, and chemosensitivity of the JFCR-39 lines to LY294002. The data for each 39 cancer cell line were plotted and were classified using the following different symbols according to their tissue types: lung (●), colon (○), gastric (▲), breast (×), ovarian (■), brain (□), renal (+), melanoma (◇), and prostate (−). The 39 experimental data points were fitted by linear regression analysis. (B) Raw protein expression profile data, including the target peaks. The three target peaks are indicated by arrows. The mass size difference between each target peak was 80 Da.

higher sensitivity to LY294002 than a cell line expressing higher amounts of the 11.6 kDa protein.

SELDI-TOF MS-based analysis for phosphorylated molecules

To verify whether the 11.7 and 11.8 kDa proteins peaks were indeed from two phosphorylated forms of the 11.6 kDa protein, we performed an *in vitro* assay using λ-PPase, which dephosphorylates all types of phosphorylated amino acid residues (i.e., p-Ser, p-Thr, and p-Tyr). Typically, the cellular proteins were incubated with λ-PPase in the absence and presence of phosphatase inhibitors for 60 min at 30 °C, applied to Q10 chips, and then analyzed by SELDI-TOF MS. Results are shown in Fig. 2. The 11.7 and 11.8 kDa peaks clearly disappeared from the reaction mixture treated with λ-PPase (Fig. 2B), but remained in the reaction mixture where the λ-PPase treatment was performed in the presence of the phosphatase inhibitors sodium orthovanadate (1 mM final concentration), sodium fluoride (25 mM final concentration), and pyrophosphate (15 mM final concentration) (Fig. 2C). These results further supported the idea that the 11.7 and 11.8 kDa molecules were single- and double-phosphorylated forms, respectively, of the 11.6 kDa protein.

Protein purification and identification of putative biomarker

To identify the 11.6 kDa protein with two potential phosphorylation sites, the MKN-1 cell lysate, which contained the 11.6, 11.7, and 11.8 kDa proteins in almost equal proportions, was used for large-scale protein purification. In order to monitor the three proteins during the purification process, the protein chips were used. First, we attempted to find an ideal condition to elute three proteins of interest from the Q10 chip using different concentrations of NaCl. We found by SELDI-TOF MS analysis that the targets remained absorbed on the Q10 chip when 200 mM NaCl was used, but were eluted off when 300 mM NaCl was used (data not shown). For large-scale purification, we absorbed cellular proteins, including the targets, onto a Q-Sepharose column and then used a NaCl

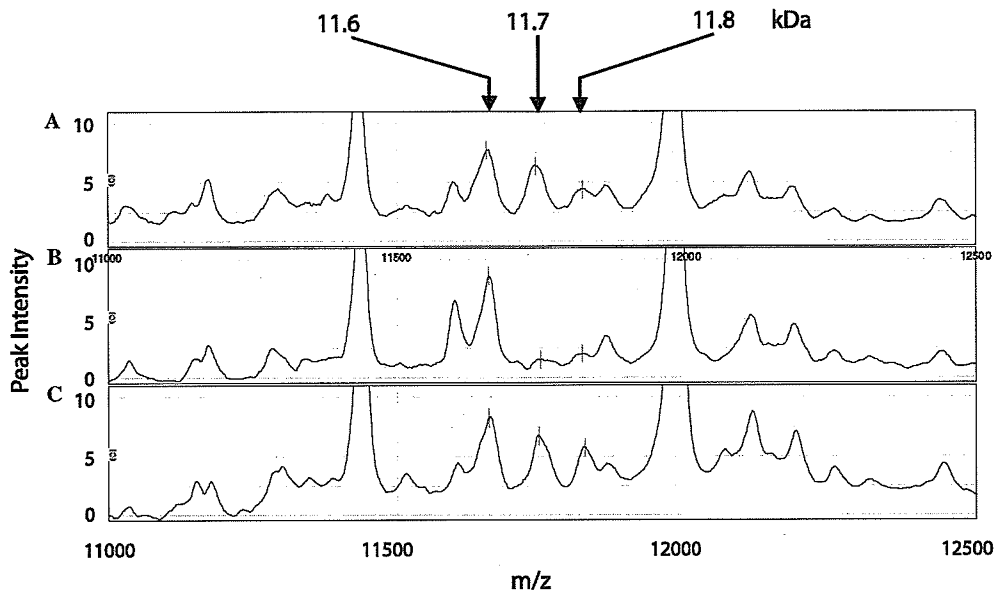

Fig. 2. SELDI-TOF MS-based analysis for determining the phosphorylation status of the target proteins. The cell lysate was incubated with: phosphatase inhibitors (A), λ-PPase (B), and phosphatase inhibitors and λ-PPase (C) for 60 min at 30 °C. Subsequently, each sample was applied to a NP20 chip and was analyzed by SELDI-TOF MS.

concentration gradient from 180 to 380 mM to elute the proteins from the column. Eluted fractions were monitored by SELDI-TOF MS using NP20 chips (Fig. 3A). The pooled fractions containing the target proteins were subjected to reverse-phase chromatography. After adsorption, the proteins were eluted with a 36–40% acetonitrile gradient, and the collected fractions were monitored by SELDI-TOF MS as before (data not shown). Since phosphorylation might inhibit protein digestion, which is necessary for the peptide-mass fingerprinting as described below, these three proteins were first dephosphorylated with λ-PPase. After dephosphorylation, we observed only the 11.6 kDa protein peak by SELDI-TOF MS (Fig. 3B). The 11.6 kDa protein was further purified on a SDS–PAGE (Fig. 3C), then the protein band was digested in-gel with trypsin or V8 protease for generating a peptide map for sequence database integration. The digestion mixture containing the peptides was applied to NP20 chips and analyzed by SELDI-TOF MS. Unique peptide sequences, obtained from the trypsin or V8 protease digestion of the 11.6 kDa protein, were entered into the Mascot search engine. Both search results identified the ribosomal P2 as the top matching protein. The probability scores using the unique fragments from the trypsin and V8 protease digestion were 63 ($P < 0.05$) and 57 ($P < 0.05$), respectively, and the sequence coverage values were 91% and 57%, respectively (data not shown). To further confirm our search results, the trypsin digested 1417.64 *m/z* fragment was analyzed by collision-induced dissociation (CID)-tandem MS with the ProteinChip Interface. We submitted the mass spectra data (masses of the product ions) to database searching using the MS-Tag search engine, and identified the peptide sequence as ILDSVGIEADDDR from the 60S ribosomal P2 (data not shown). The ribosomal P2 is a small (11,665 *m/z*) and acidic (p*I* 4.5) protein, which is consistent with the apparent molecular mass observed by SELDI-TOF MS and binding of the protein onto the Q10 chip that selectively adsorbs proteins with low p*I*s. As expected, the mammalian ribosomal P2 protein was reported to contain two prospective phosphorylation sites (S102 and S105) in the C-terminal region [17,18].

Discussion

In this study, we established a new protein expression database for the JFCR-39 lines by using SELDI-TOF MS, and combined this expression database with our previously determined chemosensitivity database to find novel biomarkers for predicting sensitivity to a PI3K inhibitor, LY294002. By integrating the two databases, we not only identified the chemosensitive biomarker candidate, ribosomal P2, but also discovered that the phosphorylation status of this protein was highly correlated with the sensitivity to LY294002. Furthermore, we demonstrated that the SELDI-TOF MS technology can be successfully utilized to isolate phosphoproteins from crude cell extracts.

The chemosensitivity database contained over 500 chemical compounds, including LY294002, and consisted of anti-cancer drugs and inhibitors [3]. Recently, we used this database to identify an *s*-triazine derivative, ZSTK474, as a novel PI3K inhibitor [6], indicating that the database was reliable and of high quality. Among those hundreds of drugs, the phosphorylation status of the ribosomal P2 also specifically correlated with the sensitivity to other PI3K inhibitors, ZSTK474 and quercetin [19], in a manner similar to that of the LY204002 (data not shown). These

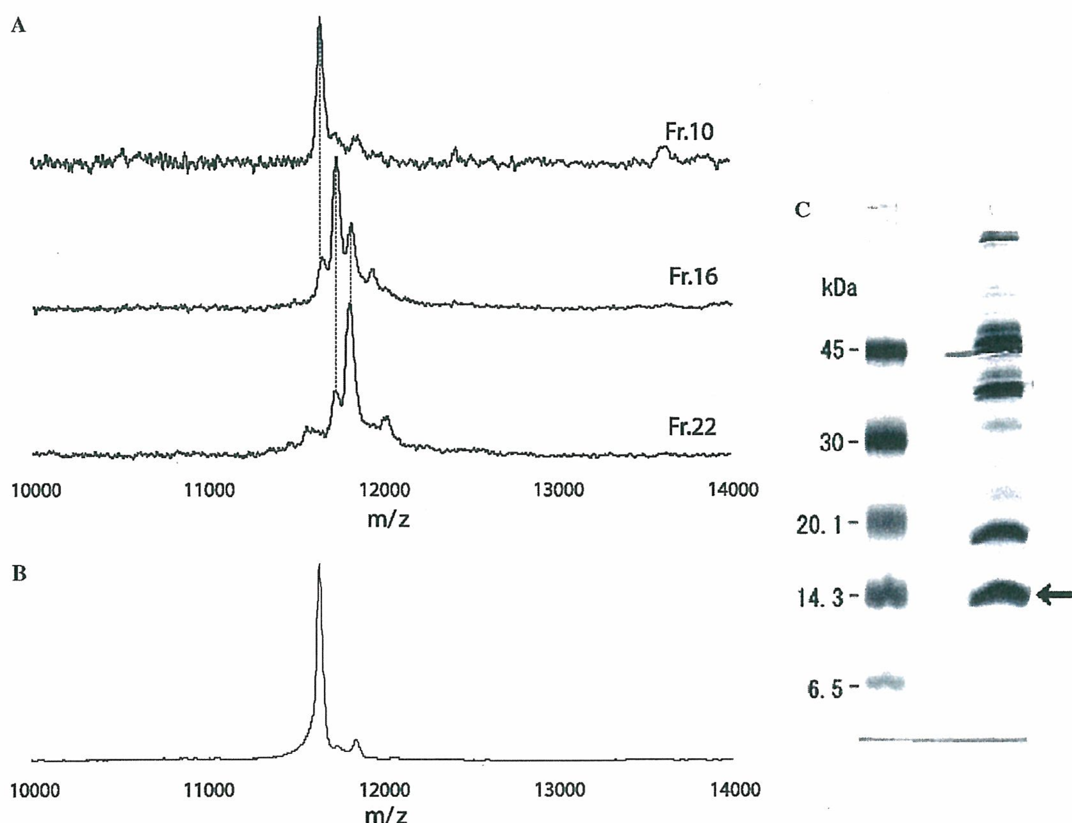

Fig. 3. Purification of the target molecules from the MKN-1 cell lysate. (A) Monitoring of elution during the Q-Sepharose column purification. Proteins from the MKN-1 cell were bound to the Q-Sepharose and eluted at the rate of 1 ml/min with 20 ml of a linear gradient of 180–380 mM NaCl in 50 mM Hepes–NaOH (pH 7.5) buffer containing phosphatase inhibitors. Eluted proteins were collected in 1 ml fractions and analyzed by SELDI-TOF MS using NP20 chips as described in the Materials and methods. Raw mass spectra of fraction numbers 10 (Fr.10), 16 (Fr.16), and 22 (Fr.22) are shown. (B) Dephosphorylation of proteins during the purification process. Proteins were fractionated using the reverse-phase chromatography, and fractions containing the 11.6, 11.7, and 11.8 kDa proteins were concentrated by SpeedVac, treated with λ-PPase, and analyzed by SELDI-TOF MS using NP20 chips. (C) Further purification of the 11.6 kDa protein on a 16.5% SDS–PAGE. The λ-PPase treated proteins were separated by SDS–PAGE and the gel was then silver-stained. The arrow indicates the 11.6 kDa protein band.

results suggested that the phosphorylation status of the ribosomal P2 might be related to the mode of action of the PI3K inhibitors.

Ribosomal P2 is a component of the eukaryotic 60S large ribosomal subunit, which forms a complex with other phosphoproteins (ribosomal P0 and P1 proteins) in the stalk region of the subunit [20]. The mammalian ribosomal P2 possesses two phosphorylation sites (S102 and S105) in the C-terminal region [17,18]. Using reconstituted ribosomes, it was demonstrated that the phosphorylated form of ribosomal P2 remarkably enhanced the protein synthesis *in vitro*, whereas the non-phosphorylated form had no effect on the protein synthesis [17]. It was also demonstrated that a reduction in the ribosomal P2 mRNA level by antisense treatment led to a change in the protein expression pattern compared with the non-treated control [21]. Together, these reports tend to suggest that protein synthesis in cells is controlled by the phosphorylation status of the ribosomal P2, and there might be a relationship between the *in vivo* protein synthesis and the sensitivity to PI3K inhibitors. However, functional significance of the phosphorylation status of the ribosomal P2 is not yet clear. Interestingly, we were able to demonstrate that the phosphorylation status of the ribosomal P2 indeed varied across the 39 cancer cell lines. As described above, cells which were less sensitive to LY294002 expressed a large amount of non-phosphorylated form of the ribosomal P2. Such abundant expression of the non-phosphorylated form of the ribosomal P2 has not been reported elsewhere. Our observations provoked a question whether there is a significant difference in function between the non-phosphorylated and the single- or double-phosphorylated forms of ribosomal P2. It was reported that phosphorylation of the ribosomal P2 increased its affinity for the elongation factor eEF-2, which plays an important role in the translation elongation [22]. Thus, phosphorylation status of ribosomal P2 may play a role in regulating the translation elongation.

The analysis of the regulatory function of phosphorylation might be important in understanding the relationship between the phosphorylation status of ribosomal P2 and the action mode of PI3K inhibitors. It is known that the

phosphorylated ribosomal S6 protein, which is a component of the 40S small ribosomal subunit and is involved in the PI3K signal pathway, efficiently regulated the protein synthesis [23], and PI3K inhibitors blocked this regulatory process [12,14]. However, at present, there is no previous report showing that the ribosomal P2 participates in the PI3K signal pathway. Ribosomal P2 was reported to be phosphorylated *in vitro* by two kinases, casein kinase 2 (CK2) [24] and G protein-coupled receptor kinase 2 (GRK2) [18]. We have found that even though the CK2 activity was strongly inhibited by LY294002 *in vitro* [25], the phosphorylation status of the ribosomal P2 did not change in several cell lines treated with LY294002 (data not shown). Since CK2 phosphorylates numerous substrates [26], implying that it may not a very specific kinase, one cannot exclude the possibility that an alternative kinase or a phosphatase might be involved in regulating the phosphorylation status of the ribosomal P2.

Detecting the phosphoprotein was beyond our expectations. Although mass spectrometry has been used previously to detect purified, phosphorylated small peptides (<5000 m/z) [27] quantitatively, there were few reports describing the detection of phosphoproteins of more than 5000 m/z size from the crude cell extract using mass spectrometry. It has been generally considered that phosphorylation reduces the ionization efficiency of the peptides, and the modified peptides are difficult to detect using mass spectrometry [28]. However, very recently, Le Bihan et al. detected an 18 kDa phosphoprotein by using SELDI-TOF MS [29]. We also successfully detected two phosphorylated forms of 12 kDa ribosomal P2 by SELDI-TOF MS in the present study. These studies may indicate that phosphorylations do not have large effects on the ionization efficiency in SELDI-TOF MS even when phosphoproteins more than 10 kDa are detected. Therefore, SELDI-TOF MS is a powerful tool because not only can it perform proteomic measurements easily, rapidly, and reproducibly [8,9], but also can be used to screen and analyze phosphoproteins from the crude cell extracts.

In conclusion, by combining the two databases we have found a new putative biomarker, ribosomal P2, and demonstrated that the phosphorylation status of ribosomal P2 was responsible for determining the sensitivity to PI3K inhibitors, especially LY294002, in the 39 cancer cell lines. We expect that the phosphorylation status of the ribosomal P2 will be a biomarker for predicting chemosensitivity. Further investigation is required to elucidate the mechanistic link between the phosphorylation status of ribosomal P2 and the chemosensitivity, and to validate this protein as a diagnostic marker.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.052.

References

[1] R.H. Shoemaker, The NCI60 human tumour cell line anticancer drug screen, Nat. Rev. Cancer 6 (2006) 813–823.

[2] J.N. Weinstein, T.G. Myers, P.M. O'Connor, S.H. Friend, A.J. Fornace Jr., K.W. Kohn, T. Fojo, S.E. Bates, L.V. Rubinstein, N.L. Anderson, J.K. Buolamwini, W.W. van Osdol, A.P. Monks, D.A. Scudiero, E.A. Sausville, D.W. Zaharevitz, B. Bunow, V.N. Viswanadhan, G.S. Johnson, R.E. Wittes, K.D. Paull, An information-intensive approach to the molecular pharmacology of cancer, Science 275 (1997) 343–349.

[3] T. Yamori, Panel of human cancer cell lines provides valuable database for drug discovery and bioinformatics, Cancer Chemother. Pharmacol. 52 (Suppl. 1) (2003) S74–S79.

[4] T. Yamori, A. Matsunaga, S. Sato, K. Yamazaki, A. Komi, K. Ishizu, I. Mita, H. Edatsugi, Y. Matsuba, K. Takezawa, O. Nakanishi, H. Kohno, Y. Nakajima, H. Komatsu, T. Andoh, T. Tsuruo, Potent antitumor activity of MS-247, a novel DNA minor groove binder, evaluated by an in vitro and in vivo human cancer cell line panel, Cancer Res. 59 (1999) 4042–4049.

[5] I. Naasani, H. Seimiya, T. Yamori, T. Tsuruo, FJ5002: a potent telomerase inhibitor identified by exploiting the disease-oriented screening program with COMPARE analysis, Cancer Res. 59 (1999) 4004–4011.

[6] S. Yaguchi, Y. Fukui, I. Koshimizu, H. Yoshimi, T. Matsuno, H. Gouda, S. Hirono, K. Yamazaki, T. Yamori, Antitumor activity of ZSTK474, a new phosphatidylinositol 3-kinase inhibitor, J. Natl. Cancer Inst. 98 (2006) 545–556.

[7] S. Dan, T. Tsunoda, O. Kitahara, R. Yanagawa, H. Zembutsu, T. Katagiri, K. Yamazaki, Y. Nakamura, T. Yamori, An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines, Cancer Res. 62 (2002) 1139–1147.

[8] N. Nakatsu, Y. Yoshida, K. Yamazaki, T. Nakamura, S. Dan, Y. Fukui, T. Yamori, Chemosensitivity profile of cancer cell lines and identification of genes determining chemosensitivity by an integrated bioinformatical approach using cDNA arrays, Mol. Cancer Ther. 4 (2005) 399–412.

[9] M. Shiwa, Y. Nishimura, R. Wakatabe, A. Fukawa, H. Arikuni, H. Ota, Y. Kato, T. Yamori, Rapid discovery and identification of a tissue-specific tumor biomarker from 39 human cancer cell lines using the SELDI ProteinChip platform, Biochem. Biophys. Res. Commun. 309 (2003) 18–25.

[10] M. Moscova, D.J. Marsh, R.C. Baxter, Protein chip discovery of secreted proteins regulated by the phosphatidylinositol 3-kinase pathway in ovarian cancer cell lines, Cancer Res. 66 (2006) 1376–1383.

[11] C.J. Vlahos, W.F. Matter, K.Y. Hui, R.F. Brown, A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), J. Biol. Chem. 269 (1994) 5241–5248.

[12] I. Vivanco, C.L. Sawyers, The phosphatidylinositol 3-Kinase AKT pathway in human cancer, Nat. Rev. Cancer 2 (2002) 489–501.

[13] Y. Samuels, K. Ericson, Oncogenic PI3K and its role in cancer, Curr. Opin. Oncol. 18 (2006) 77–82.

[14] J. Luo, B.D. Manning, L.C. Cantley, Targeting the PI3K-Akt pathway in human cancer: rationale and promise, Cancer Cell 4 (2003) 257–262.

[15] L. Stephens, R. Williams, P. Hawkins, Phosphoinositide 3-kinases as drug targets in cancer, Curr. Opin. Pharmacol. 5 (2005) 357–365.

[16] P. Workman, Inhibiting the phosphoinositide 3-kinase pathway for cancer treatment, Biochem. Soc. Trans. 32 (2004) 393–396.

[17] C. Vard, D. Guillot, P. Bargis, J.P. Lavergne, J.P. Reboud, A specific role for the phosphorylation of mammalian acidic ribosomal protein P2, J. Biol. Chem. 272 (1997) 20259–20262.

[18] J.L. Freeman, P. Gonzalo, J.A. Pitcher, A. Claing, J.P. Lavergne, J.P. Reboud, R.J. Lefkowitz, Beta 2-adrenergic receptor stimulated, G protein-coupled receptor kinase 2 mediated, phosphorylation of ribosomal protein P2, Biochemistry 41 (2002) 12850–12857.

[19] W.F. Matter, R.F. Brown, C.J. Vlahos, The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs, Biochem. Biophys. Res. Commun. 186 (1992) 624–631.

[20] M. Remacha, A. Jimenez-Diaz, C. Santos, E. Briones, R. Zambrano, M.A. Rodriguez Gabriel, E. Guarinos, J.P. Ballesta, Proteins P1, P2, and P0, components of the eukaryotic ribosome stalk. New structural and functional aspects, Biochem. Cell Biol. 73 (1995) 959–968.

[21] J. Gardner-Thorpe, H. Ito, S.W. Ashley, E.E. Whang, Ribosomal protein P2: a potential molecular target for antisense therapy of human malignancies, Anticancer Res. 23 (2003) 4549–4560.

[22] P. Bargis-Surgey, J.P. Lavergne, P. Gonzalo, C. Vard, O. Filhol-Cochet, J.P. Reboud, Interaction of elongation factor eEF-2 with ribosomal P proteins, Eur. J. Biochem. 262 (1999) 606–611.

[23] G. Thomas, M. Siegmann, A.M. Kubler, J. Gordon, L. Jimenez de Asua, Regulation of 40S ribosomal protein S6 phosphorylation in Swiss mouse 3T3 cells, Cell 19 (1980) 1015–1023.

[24] P. Hasler, N. Brot, H. Weissbach, A.P. Parnassa, K.B. Elkon, Ribosomal proteins P0, P1, and P2 are phosphorylated by casein kinase II at their conserved carboxyl termini, J. Biol. Chem. 266 (1991) 13815–13820.

[25] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, Biochem. J. 351 (2000) 95–105.

[26] F. Meggio, L.A. Pinna, One-thousand-and-one substrates of protein kinase CK2? FASEB J. 17 (2003) 349–368.

[27] E. Bowley, E. Mulvihill, J.C. Howard, B.J. Pak, B.S. Gan, D.B. O'Gorman, A novel mass spectrometry-based assay for GSK-3beta activity, BMC Biochem. 6 (2005) 29.

[28] R. Aebersold, M. Mann, Mass spectrometry-based proteomics, Nature 422 (2003) 198–207.

[29] M.C. Le Bihan, Y. Hou, N. Harris, E. Tarelli, G.R. Coulton, Proteomic analysis of fast and slow muscles from normal and kyphoscoliotic mice using protein arrays, 2-DE and MS, Proteomics 6 (2006) 4646–4661.

Special Review

Percellome Projectによる毒性トランスクリプトミクスの新しい試み

Percellome Project as a New Approach to Toxicology Transcriptomics

菅野 純　北嶋 聡　相﨑健一　五十嵐勝秀　中津則之　高木篤也　小川幸男　児玉幸夫
Jun Kanno, Satoshi Kitajima, Ken-ichi Aisaki, Katsuhide Igarashi, Noriyuki Nakatsu, Atsuya Takagi, Yukio Ogawa, Yukio Kodama

身の回りの物質の毒性（有害性）を予測し，その被害を未然に防ぐのが毒性学の役割である．この精度向上を目指したトキシコゲノミクス研究を実施する際に，マイクロアレイなどから細胞1個当たりのmRNAコピー数を得るPercellome法を開発した．90化合物のマウス肝初期応答データを採取し終え，新たな対象（反復投与，胎児毒性，吸入毒性，多臓器連携）を加えたPercellome Projectを展開している．

key words
トキシコゲノミクス，分子毒性学，遺伝子発現カスケード，標準化，Percellome法，3次元多層(Millefeuille) データ

i 菅野 純　国立医薬品食品衛生研究所 安全性生物試験研究センター 毒性部　E-mail：kanno@nihs.go.jp
1985年東京医科歯科大学大学院医学研究科博士課程修了．人体病理学，実験病理学専攻．国立医薬品食品衛生研究所毒性部室長を経て，2002年より同部長．内分泌かく乱関連などの分子毒性学研究，トキシコゲノミクスプロジェクトなどを厚生労働所掌業務との有機的連携のもとに推進．

北嶋 聡，相﨑健一，五十嵐勝秀，中津則之，高木篤也，小川幸男，児玉幸夫　国立医薬品食品衛生研究所 安全性生物試験研究センター 毒性部

はじめに

医薬品，食品，化粧品，生活関連用品など，身の回りの物質が我々の身体に取り込まれた際に生じる可能性のある毒性（有害性）を予測し，それらの使用に際しての被害を未然に防ぐのが毒性学の役割である[注1]（図1）．具体的には，人々の安全を確保するために使用法（用途）や使用量（残留量）を制限したり，場合によっては禁止したりするための科学的根拠を提供するが，その際，人の身代わりとして実験動物を用いる場合が多い．このような毒性学の精度向上の一環として，従来からの毒性研究（毒性症候学，毒性病理学，など）に加えてのトキシコゲノミクス（Toxicogenomics）研究が進められている．

トキシコゲノミクスでは，物質が生体に及ぼす影響をトランスクリプトームとして観測・解析する．その際，①分子毒性学を構築し種差や個体差の問題，複合暴露の問題などを解決するためには，遺伝子発現カスケードの全容解明を目指す必要がある，②形態学的に変化が現れた段階のトランスクリプトームは，遺伝子発現カスケードの最終段階に過ぎない，③形態変化の現れないごく初期段階を含む遺伝子発現カスケードを描出するためにはまとまった量のデータの蓄積が必須である，との観点から，筆者らは，マイクロアレイや定量PCRから細胞1個当たりのmRNAコピー数を得るPercellome手法と，そのデータ解析のための3次元多層（Millefeuille）システムを開発・実用化した．遺伝子発現量が共通の尺度，すなわち“コピー数／細胞”で表現されることから，検体間，実験間，マイクロアレイのバージョン間，異なったプラットホーム間，などのデータ比較が直接的に行えるようになり，数年かけて蓄積したデータの有機的活用が可能となった．現在，90種類の化学物質によるマウス肝の初期応答データを採取し終えたところである．新たな対象（反復投与，胎児毒性，吸入毒性，多臓器連携）を加えたPercellome Projectの概要を紹介する．

注1　環境への配慮も含まれる．

Ⅰ．Percellome法：細胞1個当たりのmRNA絶対量を得る方法

原理は単純である．サンプルの細胞数を計測し，外部標準mRNA（スパイクRNA）を細胞1個当たり決まった分子数だけそのサンプルに添加し，そしてRNA抽出，測定に移る．サンプルのRNAの測定値を，スパイクRNAの値を基準に，細胞1個当たりのコピー数に換算する．実際には細胞数を直接計測するのが困難なことが多いため，その代替指標として細胞核内のゲノムDNA量を用いる[1), 2)]．定量性・直線性の検証にはLBM標準サンプル（肝[L]と脳[B]を100：0，75：25，50：50，25：75および0：100に混合した5サンプルから成るセット）を用いる．なお，スパイクRNAは，5種類の枯草菌遺伝子のmRNAを濃度公比3で混合したカクテル（dose-graded spike cocktail；GSC）として用意した．高精度を要求されるDNA定量法は手作業プロトコールおよび自動ロボット（PerkinElmer JANUS）のプロトコールを準備

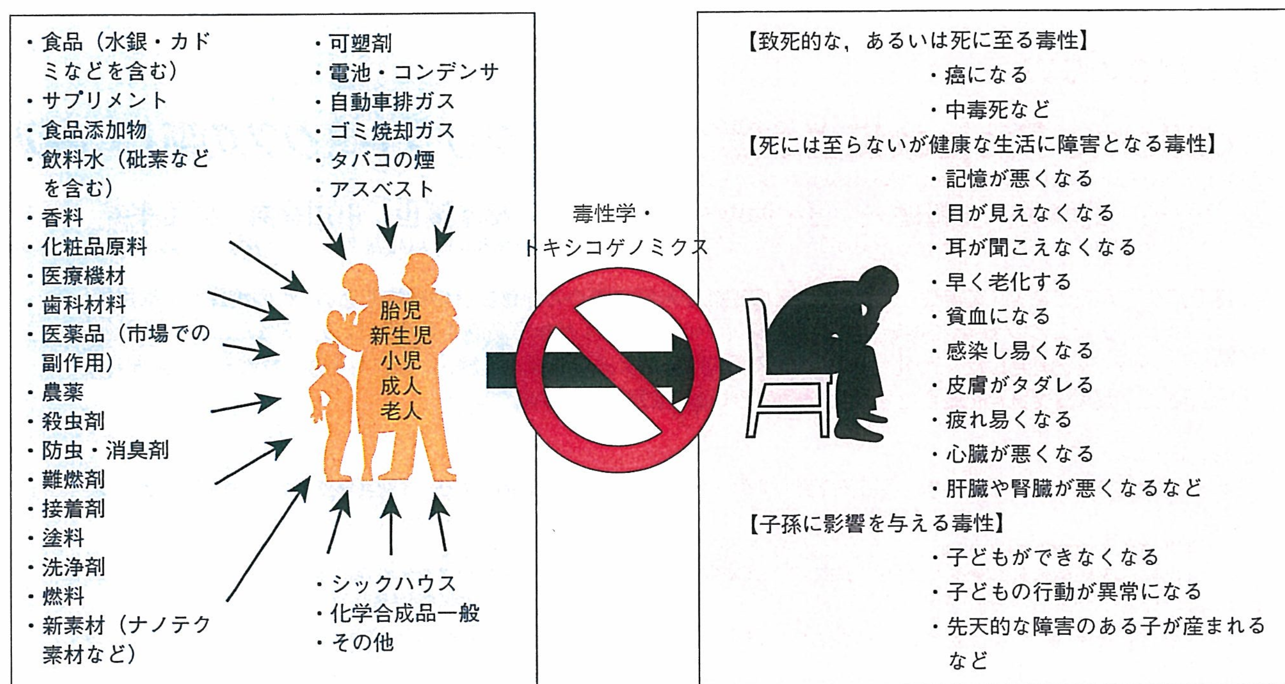

図1. 毒性学の対象

毒性学は，身の回りの物質が引き起こす障害を予測し，その発生を未然に防ぐことを目的としている．トキシコゲノミクス（毒性ゲノミクス）は，最先端の網羅的遺伝子発現解析技術を用いて，従来の毒性学の予測の精度を著しく向上，迅速化させることで，国民の健康安全の確保にさらに貢献することを目指している．

中である．カクテルとも共同研究ベースで供給可能である（連絡先：kanno@nihs.go.jp）．また，ERCC（The External RNA Control Consortium）と連絡をとるとともに，国際的標準化への関与を深めるため平成18年度厚労科研費「医薬品などの有効性・安全性評価に資する遺伝子発現解析の国際的標準化に関わる研究（H18-特別-指定-023）」を立ち上げた．現在，この他にシックハウス症候群を考慮した低用量域での吸入毒性トキシコゲノミクス，1匹のマウスから多臓器を採取しそれらの連携状況をトランスクリプトームから解析する多臓器トキシコゲノミクスを開始し，特徴的な遺伝子について組織内の発現分布を *in situ* ハイブリダイゼーションで確認する作業を並行している．また，下記の3次元データをweb公開するサーバを整備し，一部の化合物から3次元多層（Millefeuille）データを順次閲覧可能とした（http://toxicomics. nihs.go.jp/db/）．

Ⅱ. 3次元多層（Millefeuille）データシステム：生物系研究者に優しいデータ可視化と解析

医薬品を含む毒性既知の90化合物について単回経口投与後のトランスクリプトームデータを取得して，初期応答遺伝子カスケードを解析するための基盤データベースを構築した．現在，第二段階として反復暴露データ集積を開始した．データは，用量軸，時間軸，および遺伝子発現軸から成る3次元表示により，遺伝子発現の用量および時間に依存した変化を1枚の曲面として表すことで可視的に変化を判別しやすいように配慮した（図2）．これにより，コンピュータが選び出した遺伝子クラスターの中身を確認する際，特に，mRNAの合成分解のスピードなどの知見から生物学的にありえないパターン（用量軸の方向にも時間軸の方向にもジグザグな変化など）を排除する際に威力を発揮している．

1つの実験から排出されるGeneChip約50枚のデータを一括処理する能力を持ったPercellome自動換算・データ品質管理（QC）に関わるソフトウエアに加えて，3次元多層（Millefeuille）データに最適化した，発現パターン類似性による候補遺伝子検索，およびそれを発展させた教師無しクラスタリング[3]を中心とした解析システム（MF System，MFシリーズ，開発：相﨑 健一）を独自に実用化し，開発継続中である（図3）．これらにより，データQCはその日のうちに，基本的な発現情報検索から全遺伝子の教師無しクラスタリングまでを3日間で完遂できるものとなっている．

この基本解析を用いて，発現パターンによって分類された候補遺伝子リストが多数生成される．一部の幸運な例ではただちに新規と思われる毒性関連反応を見いだすことができた．またそうでない場合のための1つの補強手段とし

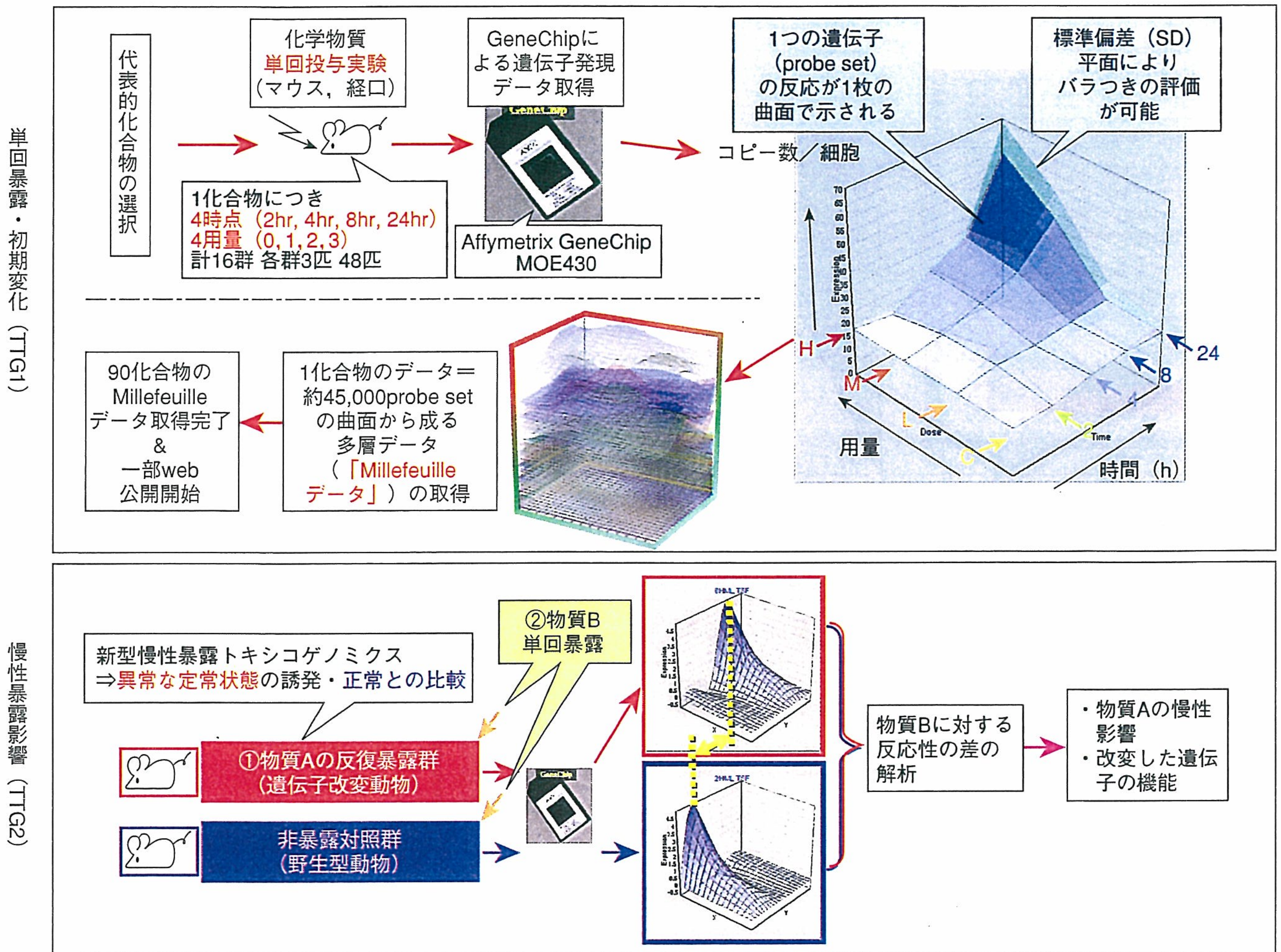

図2. Percellome法と3次元表示による多層（Millefeuille）データシステムを用いたプロジェクトの根幹部分の概要
単回投与による遺伝子発現初期変化データを90化合物について取得（上段）．現在，反復投与の影響を検討中（下段）．H；高用量（high），M；中用量（medium），L；低用量（low），C；コントロール（control）．

て，Gene Ontologyなどの既存知識を利用して候補遺伝子リストの理解を支援するソフトウエア（MF GoPlot）を用意した．このツールは一種の化合物クラスタリングとしても利用することができる．

さらに候補遺伝子リストを基に複数化合物間比較を行い，複数条件下においても同期して発現する遺伝子群を自動抽出するシステムも開発済みである．本システムで得られた同期遺伝子群はシグナルカスケードの構成単位である可能性があり，データベース化しつつ，その解析を進めている（5TB規模のデータベース部分および，大量計算アルゴリズム実装は（株）NTTコムウェアおよび（株）日本NCR/Teradataとの共同開発による）．

Ⅲ. Percellome手法のリアルタイムPCRを含む他のプラットホームへの適用

Percellome手法は，GSCの受け入れ条件を整えることにより，様々なプラットホームに適用可能である．その1つとして最も定量性が高いとされるリアルタイムPCR（ABI PRISM 7900 HT・96ウェルプレート）への適用例を示す．現行のRT-PCR絶対定量法では，遺伝子ごとに検量線が必要であり，多数のサンプルについて多数の遺伝子を検討するには不向きである．Percellome RT-PCRでは，マイクロアレイと同様の原理を用いる．すなわち，サンプル破砕液に，その細胞数に比例する量のスパイクカクテル（GSC）を添加し，それらのCt値をPCRプレートごとの検量線とすることにより，測定したい遺伝子のCt値を細胞1個当たりのmRNAコピー数に換算する．これにより，GAPDHやActinなどのハウスキーピング遺伝子が変動してしまう際の問題，例えば，少数の遺伝子を検討する際にGlobal normalization法を適用し難い問題などが解決される．共通サンプルを測定しデータを比較することにより，Affymetrix GeneChipのPercellome結果と9割程度の整合性が確認され，

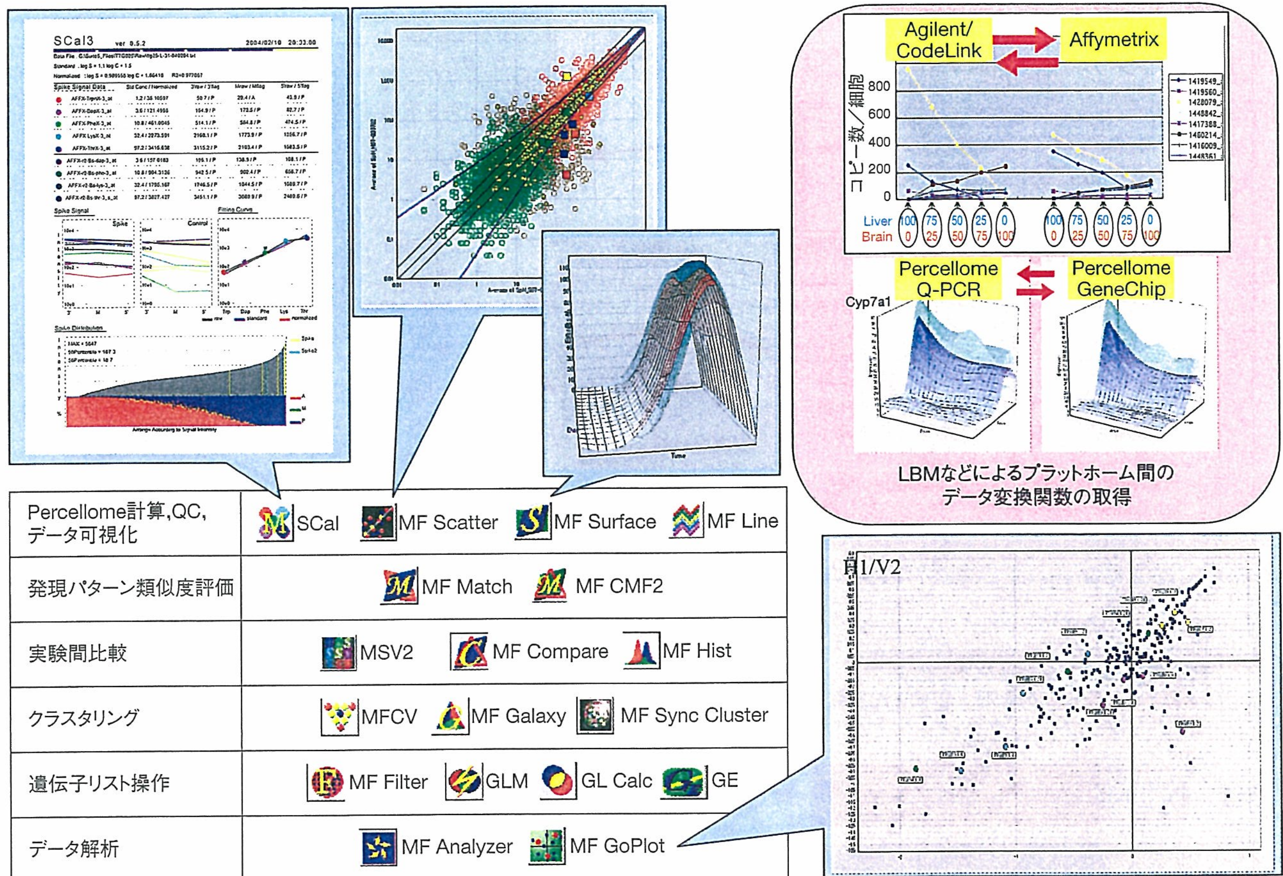

図3．3次元多層（Millefeuille）データの解析などに用いる独自開発プログラム群

品質管理とともにPrecellome計算を自動的に実施するSCal，Plotソフトウエア，3次元曲面の描画ソフト（MF Surface），など．右上はプラットホーム間のデータ変換情報の得方を示す．LBMを用いる方法（上段）と，実際の実験サンプルを用いる方法（下段）がある．いずれも，一度，両方のプラットホームでそれらのサンプルを測定する必要がある．

GeneChipとPercellome RT-PCRとの間でのコピー数の換算式がいくつかの遺伝子について得られている．この他に，Agilent社製の単色マイクロアレイとCodeLinkアレイにGSCを測定可能なカスタムアレイを用意し終え，LBMサンプルのデータなどをもとに，これらとの間の換算式も得つつある（図3右上）．

Percellome法は，Affymetrixの新しいエキソンアレイの定量性・直線性の検討にも適応可能である．Affymetrix社のHuman Exon 1.0 ST Arrayと従来型の発現アレイHuman Genome U133 plus 2について，性質の異なるヒト癌細胞株2株から調製したLBM様標準サンプル（100：0，75：25，50：50，25：75および0：100混合5サンプル）による比較を行い，両アレイ間の相関性の高いprobe setを多数検出することができた．また，既知のエキソンに対して設計されたprobe setでは発現が見られ，イントロンに対して設計されたprobe setでは発現が見られない，あるいは，既知のsplicing variantに対応したprobe setの発現が検出された，などの基本性能が確認された．しかし，Percellome法を適用して未知のsplicing variantの検出力を向上させるためには，現状では各エキソン間の定量性に問題があることが示唆された．定量値を算出する補正アルゴリズムの開発など，何らかの対策が必要であることが考えられ，現在，Affymetrix社に確認を行っている．

Ⅳ．核内受容体原性毒性のPercellomeトキシコゲノミクス解析

受容体原性毒性とは，化学物質が受容体（リガンド依存的転写因子を含む）に選択的に結合してシグナルをかく乱し，その結果生じる有害性を指す．代表例としてはダイオキシンが挙げられる．AhR（Arylhydrocarbon receptor）ノックアウトマウスでは，ダイオキシンを大量に投与しても毒性がほとんど観察されない．すなわち，野生型マウスがダイオキシンで死ぬメカニズムには，AhRが必須であり，AhRからの異常なシグナルがマウスを死に至らせていることに

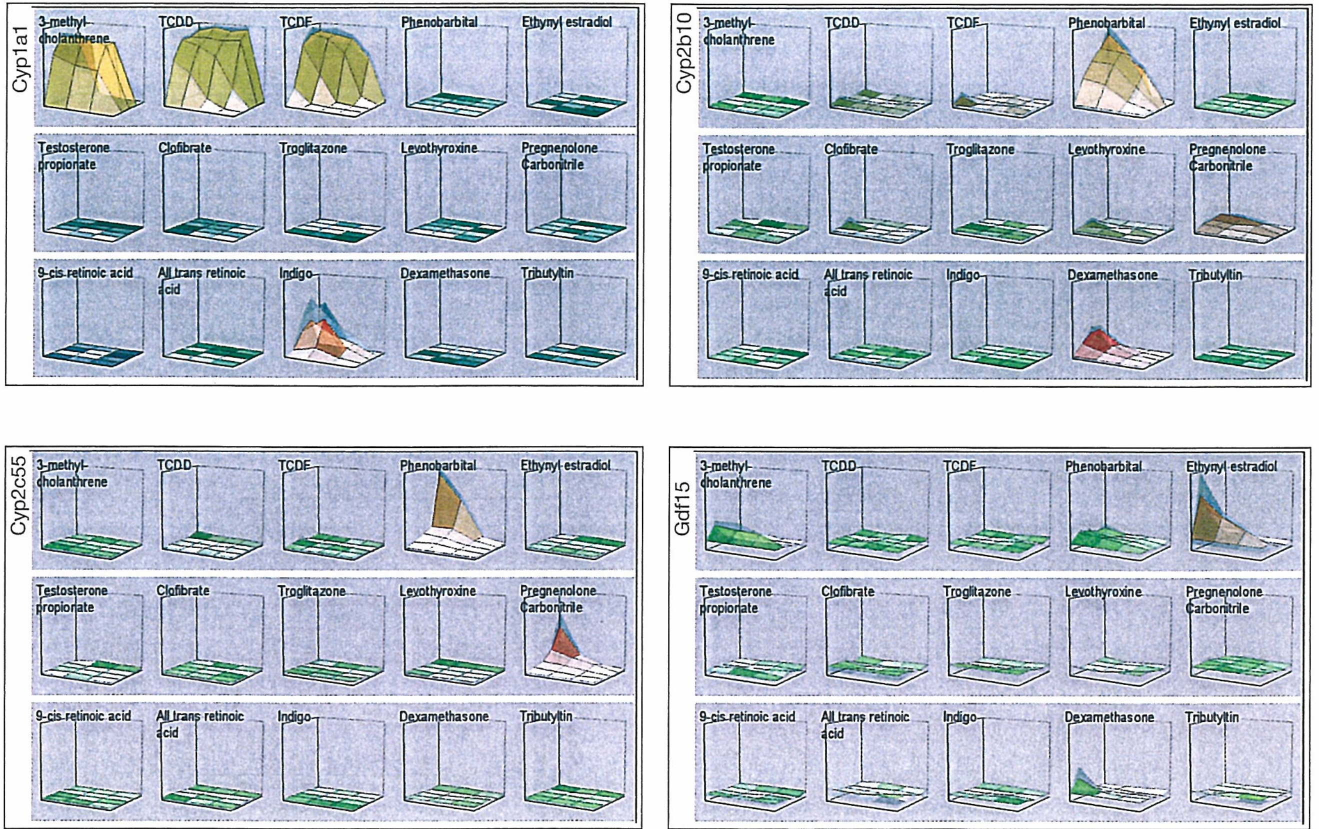

図4. 化合物間の発現比較

15種類の核内受容体リガンド化合物（各3次元グラフ内に表示）によるCyp1a1（左上），Cyp2c55（左下），Cyp2b10（右上）および，Gdf15（右下）の遺伝子発現を3次元表示したもの．各軸は，図2のとおり．縦軸のスケールは遺伝子ごとに共通．リガンドに選択的な遺伝子の発現が確認される．

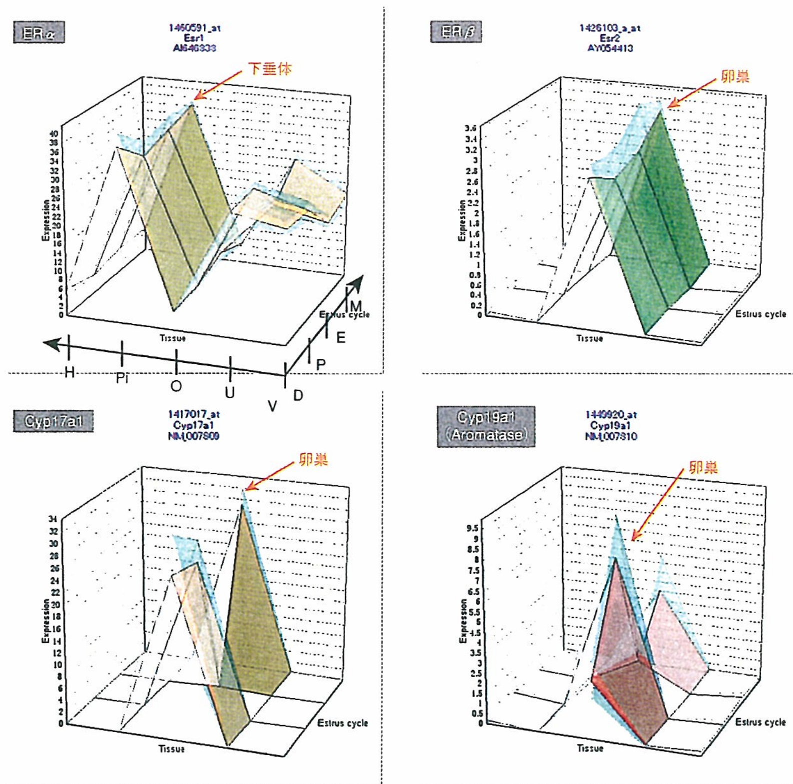

図5. 臓器間の発現比較

マウスの性周期（Diestrus, Proestrus, Estrus, Metestrusの4日間で1周期）ごとの視床下部（H），下垂体（Pi），卵巣（O），子宮（U）および膣（V）における，ER α，ER β，Cyp17a1（steroid-17 α -hydroxylase），およびCyp19a1（Aromatase）の遺伝子発現変動を3次元表示したもの．後二者の酵素は卵巣において周期性を持って発現している．

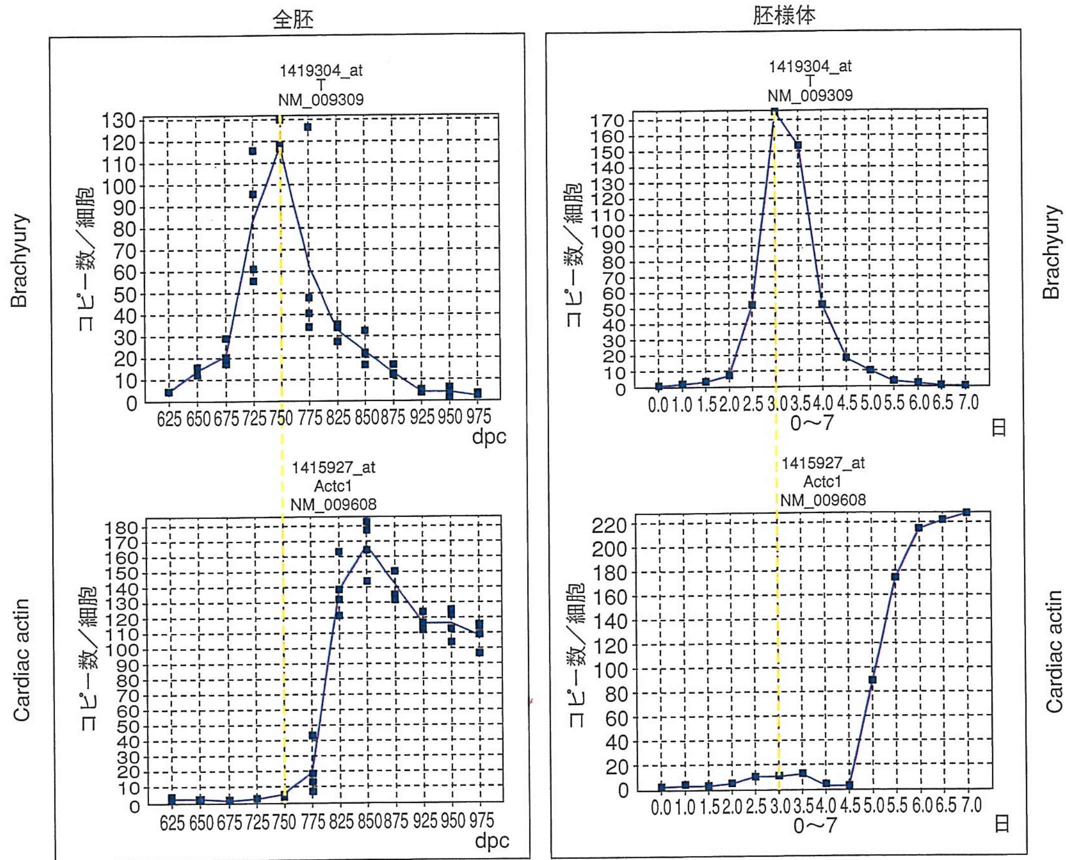

図6. マウス胎児（全胚）と胚様体の発現比較

マウス全胚の胎生6.25日～9.75日までの遺伝子発現と，胚様体の1日～7日目までの遺伝子変動の網羅的データベースから，初期中胚葉分化マーカーであるBrachyury遺伝子と，Cardiac actin遺伝子の経時変化を示す．

なる．エストロジェン活性化学物質による有害影響（内分泌かく乱化学物質問題）も同様にER（estrogen receptor）を介する受容体原性毒性と考えられ，胎生期にERを発現する組織が，低用量シグナルかく乱影響の重要標的であると考えられている．

ここでは，受容体原性毒性研究の基盤として，Percellome手法を適用して，①核内受容体作動性物質によるマウス雄肝臓の遺伝子発現変動，②性周期に伴うマウス雌生殖器遺伝子発現変動，③生後の発達過程におけるマウス雌生殖器遺伝子発現変動，の3種類のデータベースを構築した．例えば，①では10種類の核内受容体に作用する典型物質について，単回経口投与後，2，4，8，24時間目の変動を解析し，Ethynyl-estradiolがGDF15，TCDDがCyp1a1，9-cis Retinoic AcidがCyp26a1，DexamethasoneがCyp2b10，ClofibrateがCyp4a14，PCNがCyp2c55など，各々の受容体に特徴的な遺伝子発現を誘導するところがとらえられている（図4）．②の性周期データベースは視床下部，下垂体，卵巣，子宮，腟を対象としており，性周期との関連が網羅的にとらえられている（図5）．これらのデータベースは，今後，各種の候補物質が引き起こす変化を詳細に解析する際の基準として利用される．

V．発生トキシコゲノミクスへの応用

発生毒性学は，個体発生過程におけるダイナミックな遺伝子発現調節の分子機構を把握することにより，さらに正確なものに補強されると考える．現在，C57BL/6マウス胚の器官形成期初期にあたる胎生6.5～9.5日（プラグ確認日：0.5日）の，①全胚の遺伝子発現変動解析，②遺伝子欠失マウス全胚との比較，および③標的が明らかな既知発生毒性物質投与による本データベースの具体的な適用，を実施している．①についてはすでに0.25日間隔（Time point 計12点）の遺伝子発現データベースを得て，②遺伝子欠失胚のデータといくつかの注目すべき遺伝子についてはwhole mount ISHを用いた発現の検証を加えた．これと並行して，ES細胞からhanging drop法で得た胚様体の0.5日間隔の遺伝子発現データとの比較を実施している．個体発生に関与

する遺伝子群の多くは経時的に激しく変化しており，既知発生毒性物質投与実験については標的遺伝子シグナルカスケードを解析中である（図6）．

おわりに

ノーザンブロットでは実験サンプルにだけバンドが見られ，対照サンプルには遺伝子発現がないという結果を得ても，細胞1個当たりで定量してみると，対照が10コピーに対して実験サンプルが20コピーである場合がある．“無”が“有”になったのではなく，“10”が“20”になったのである．

さて，筆者らの属する毒性学でも，医学の分野でも，疾患概念や毒性概念が整理され，患者や実験動物を診断する際には，まず，そのどれに当てはまるかを検討する．すなわち，どの“典型”に近い症例であるかを検討することから始まることが多い．

しかし，最近の医学・生物学には多因子疾患・多因子形質発現制御の概念が導入され，今から何年かの後には，“21世紀初頭までは，患者の遺伝子多型を調べずして治療を行っていた時代”として，“血液型を調べずに輸血していた時代”と並び称されるようになる可能性がある．このような多因子概念が定着すると，その多くは，“有（100%）”“無（0%）”の組み合わせではなく，“70%”“50%”“90%”といった半端な数の組み合わせであることが考えられる．すなわち，今までの離散値的な“典型”例を基準とするアプローチから，連続値的な病態“スペクトラム”を直接扱うアプローチに変革していく可能性が考えられる．その際の網羅的データの解析とその蓄積の必要性を考えるとき，遺伝子発現データの定量化・標準化という問題は，今まで以上に重みを増すと考えられる．生命現象の網羅的解析にはトランスクリプトームだけでは不十分であることは自明であるが，この定量性を確保することは，これから実現されるであろう網羅的プロテオミクスなどの基盤としても重要ではないかと考える．

マイクロアレイなどから得られるトランスクリプトーム情報が，今後の医薬品審査や化学物質の安全性評価の際に必須なものとなる時代がすぐそこまで来ていることを念頭に，筆者らはPercellome法をさらに展開し，Percellome Projectデータベースを可能な限り高精度に保ちつつ毒性学的な内容を充実させるべく最大限の活動を継続して行く所存であるが，この技術，あるいは研究内容が毒性学以外の研究分野にもお役に立つことができれば幸甚である．

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– 文献

1) Kanno J, et al: BMC Genomics (2006) 7: 64
2) 菅野 純ら: 細胞工学 (2004) 23: 685-693
3) Matsumoto S, et al: Genome Informatics (2005) 16: 183-194

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Pediatric susceptibility to 18 industrial chemicals: A comparative analysis of newborn with young animals

R. Hasegawa [a,*], M. Hirata-Koizumi [a], M. Dourson [b], A. Parker [b], A. Hirose [a], S. Nakai [c], E. Kamata [a], M. Ema [a]

[a] *National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Toxicology Excellence for Risk Assessment, 2300 Montana Avenue, Suite 409, OH 45211, USA*
[c] *Yokohama National University, 79-7 Tokiwadai, Hodogaya, Yokohama 240-8501, Japan*

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Abstract

We comprehensively re-analyzed the toxicity data for 18 industrial chemicals from repeated oral exposures in newborn and young rats, which were previously published. Two new toxicity endpoints specific to this comparative analysis were identified, the first, the presumed no observed adverse effect level (pNOAEL) was estimated based on results of both main and dose-finding studies, and the second, the presumed unequivocally toxic level (pUETL) was defined as a clear toxic dose giving similar severity in both newborn and young rats. Based on the analyses of both pNOAEL and pUETL ratios between the different ages, newborn rats demonstrated greater susceptibility (at most 8-fold) to nearly two thirds of these 18 chemicals (mostly phenolic substances), and less or nearly equal sensitivity to the other chemicals. Exceptionally one chemical only showed toxicity in newborn rats. In addition, Benchmark Dose Lower Bound (BMDL) estimates were calculated as an alternative endpoint. Most BMDLs were comparable to their corresponding pNOAELs and the overall correlation coefficient was 0.904. We discussed how our results can be incorporated into chemical risk assessment approaches to protect pediatric health from direct oral exposure to chemicals.
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Keywords: Pediatric susceptibility; Industrial chemicals; Phenols; Newborn rats; Childhood exposure; Uncertainty factors; ADI; TDI; Benchmark dose

1. Introduction

Exposure of humans to environmental chemicals may occur via several routes such as the mouth, respiratory system, skin and eyes. As a result, regulatory/limit levels in food, water and air have been established to protect human health through risk assessment, which is usually based on toxicity data from animal studies (Hasegawa et al., 2004). However, the early postnatal period, especially the nursing phase, is not directly covered by current risk assessment approaches because of the inherent lack of toxicity information. Rather, two uncertainty factors are used to cover this data gap, one for human variability to toxic insult and the other for the lack of specific data to determine the critical effect (Dourson et al., 2002).

Repeated-dose oral rodent studies administer chemicals starting at approximately six weeks of age (OECD, 1995). In two-generation toxicity studies, chemicals are usually fed to rodents during the entire experimental period but newborn animals are only exposed to chemicals indirectly through maternal milk during nursing (up to 3 weeks old), or through small amounts of foods containing chemicals at about day 14 or older (OECD, 2001). Thus, there is generally no definitive toxicity information for chemical exposure in newborn animals.

Human infants may ingest not only baby foods and liquids but also household materials, fluids, and soil. They have unique physiological characteristics with regard to their organ/body balance, and the immature structure

* Corresponding author. Fax: +81 3 3700 9788.
E-mail address: hasegawa@nihs.go.jp (R. Hasegawa).

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and functions of various organs may lead to elevated susceptibility or sensitivity (Scheuplein et al., 2002; Polin et al., 2004). Even though newborn exposure studies cannot be conducted for all chemicals due to ethical limitations, limited human and economic resources, or handling difficulties, such studies are valuable for the assessment of pediatric health risk given the appropriate comparative attention now being drawn to infant and child health world wide (Landrigan et al., 2004; IFCS, 2005).

Therefore, we have established an 18 day repeated-dose newborn rat toxicity study protocol, and conducted newborn studies for 18 industrial chemicals using this protocol, although the selected chemicals were mostly limited to phenolic compounds due to financial support. In addition, we have compared the newborn results with the results of a 28 day repeated-dose study (young study) and published all of the detailed analysis in peer-reviewed journals (Koizumi et al., 2001, 2002, 2003; Fukuda et al., 2004; Takahashi et al., 2004, 2006; Hasegawa et al., 2005; Hirata-Koizumi et al., 2005a,b).

In this article, we compare the results of these published studies by first describing our comparative study conditions common to all chemicals, then providing a summary of the final re-analyzed data, and finally discussing how our results can be incorporated into chemical risk assessment approaches to protect pediatric health from direct oral exposure to chemicals.

2. Experimental conditions of newborn and young studies for comparison

To appropriately elucidate differences in chemical sensitivity, studies in newborn and young rat were conducted under the same experimental conditions as closely as possible. For example,

(1) Sprague-Dawley SPF rats [Crj:CD(SD)IGS] purchased from Charles River Japan Inc. (Yokohama, Japan) were used for all studies;
(2) the same Lot Number for each chemical was used for both newborn and young studies;
(3) test solutions were prepared by the same methods with the same vehicles for both studies and administered by gastric intubation;
(4) test solutions were prepared at least once a week and kept cool and in the dark until dosing; stability was confirmed to be at least 7 days under these conditions; and
(5) all other reagents used in this study were specific purity grade;
(6) all animal treatments were conducted in 5 Japanese contract laboratories according to their Animal Care Guidelines and Japanese GLP Guidelines inspected by the Government.

The only differences in conditions were the administration period of 18 days for newborn and 28 days for young rats, and the recovery (maintenance) period as described in Fig. 1. Since rearing conditions for newborn rats change abruptly from nursing by foster mothers to individual self-feeding at postnatal Day 21 it was considered to be the best termination time point for the newborn dosing (a dosing period of 18 days) rather than adopting the same dosing period for the young studies (28 days).

2.1. Young studies

All schedules and examinations were performed in compliance with the Test Guideline "28 day repeated-dose toxicity study using mammals" of the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances). This guideline is equivalent to OECD Test Guideline 407.

A dose-finding study was conducted according to the results of a single oral toxicity study. The study had a shorter dosing period (14 days) when compared to the main

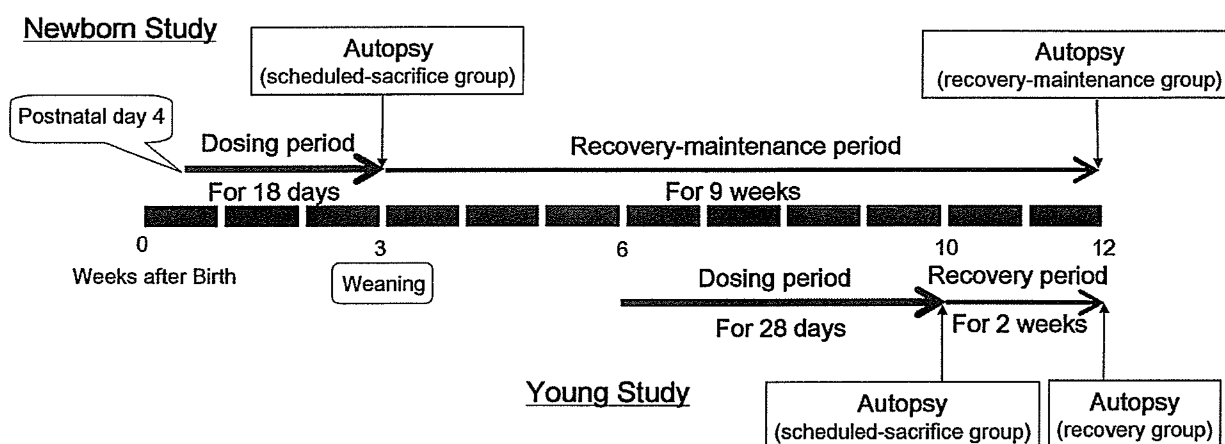

Fig. 1. Dynamic comparison of schedules for newborn and young studies.

study, and included most examination, but did not have examination of histopathology, urinalysis or recovery groups.

In the main study, at least 5 rats of both sexes were assigned to control, low, medium and high dose groups, and at least 5 rats of both sexes were assigned to control and high doses as recovery groups. Animals at 5–6 weeks of age were administered chemicals by gastric intubation daily for 28 days and sacrificed under ether anesthesia following the last treatment after overnight starvation (scheduled-sacrifice group). The recovery groups were maintained for 2 weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. Observation of general behavior and estimation of body weight and food consumption were conducted during dosing and recovery periods. Macroscopic findings, blood chemistry (20 items), hematology (10 items), urinalysis (11 items), organ weights (15 organs) and histopathology (18 organs) were examined for the sacrificed animals.

2.2. Newborn studies

We established a newborn rat study protocol due to the lack of any standard test guideline for newborn animals. Fig. 1 shows the dosing and examination schedule for the newborn rat and young rat study. Pregnant rats (gestation day 14) were purchased and allowed to deliver spontaneously.

For a dose-finding study, all experimental conditions including the administration period were the same as the main study described below except that no examination of histopathology or urinalysis occurred, and no recovery-maintenance groups were maintained.

In the main study, dosing began on postnatal Day 4 with the administration of chemicals to 12 males and 12 female pups in each of 4 groups (control, low, medium and high doses). Each littermate consisted of 4 male and 4 female pups given different dose of chemical. Dosing to the pups continued up to weaning on postnatal Day 21 (18 days). On postnatal Day 22, half of the pups in each group were sacrificed under ether anesthesia (scheduled-sacrifice group), and remaining pups in all groups were maintained for 9 weeks without chemical treatment and subsequently sacrificed at 12 weeks of age (recovery-maintenance group). Observation of behavior and estimation of body weight and food consumption were conducted as with the young rat study protocol. The groups were examined for developmental parameters such as surface righting and visual placing reflexes for reflex ontogeny; fur appearance, incisor eruption and eye opening for external development during dosing period; and sexual development such as preputial separation, vaginal opening and estrous cycle during the recovery-maintenance period. The long recovery-maintenance period allowed for examination of sexual development after weaning and latent toxic effects in the early adulthood.

3. Unique approach to analysis of the susceptibility of newborn rats to chemicals

The no observed adverse effect level (NOAEL) is frequently used to determine safety or toxicity for environmental and industrial chemicals, with the NOAEL being the greatest dose at which no adverse effects are observed. However, the NOAEL is not always appropriate for an accurate comparison of toxicity levels between studies because the NOAEL is dependent on the dose setting. For example, in our early analysis of 2,4-dinitrophenol data, NOAELs for both newborn and young rat main studies were both 10 mg/kg/day because clinical signs of toxicity appeared at 20 mg/kg in newborn and 30 mg/kg in young rats. However, newborn rats seemed to be more sensitive to the chemical considering the intensity of lesions at higher doses. Further analysis of the data from the dose-finding young study showed no clinical toxicity signs at 20 mg/kg. Therefore, 20 mg/kg/day from the dose-finding young study was considered to be more appropriate as a NOAEL than the 10 mg/kg/day from the main young study. Including the dose-finding study in the determination of the NOAEL for a main study is not commonly done, thus, we decided to employ a new terminology in this document; the presumed NOAEL (pNOAEL) and defined it as the most likely no adverse effect dose for our specific purpose. The lack of information from dose-finding studies, such as histopathological examination in both newborn and young studies, and the shorter administration period in the young case was carefully considered in adopting the pNOAEL approach.

In addition, a Benchmark Dose (BMD) approach was applied to the same toxicity endpoint data that was used for the estimation of pNOAEL. Although clinical signs and histopathological changes are generally not appropriate for BMD analysis, since the frequency, duration and severity cannot usually be incorporated for the calculation, we attempted to employ incidences such as numbers of affected animals from both main and dose-finding studies where appropriate. Using the US EPA provided Benchmark Dose Software (Version 1.3.2), Benchmark Dose Lower Bound (BMDL) was estimated with 10% extra incidence at the 95% confidence level. In most cases, the incidence data were input to a Dichotomous model. For selection of the model, the lowest AIC (Akaike's Information Criterion) was used and the goodness-of-fit was confirmed visually with graphical displays.

At the first trial to evaluate the susceptibility of newborn rats to chemicals, we judged that the above endpoint comparison of pNOAEL/BMDL was not sufficient with respect of outcome reliability and the full toxicity data set should have been used. Alternatively, comparison of pNOAEL/BMDL might be sufficient for low dose responses but not with results at Lowest Observed Adverse Effect Level (LOAEL). In fact, it is reported that 17-day-old rats show higher susceptibility to chlorpyrifos at the maximum tolerated dose than adult rats (Moser and Padilla, 1998),

whereas no differential sensitivity was evident in NOAELs between the two groups (Pope and Liu, 1997)—a fact repeated with several chemicals in our series. Thus, we also considered the comparison of LOAELs among the newborn and young animal studies. Unfortunately, the traditional comparison of LOAELs has frequently suffered from a disparity in severities among studies, a situation that continues today with comparison of BMDLs.

This lead us again to employ a new terminology; the presumed unequivocally toxic level (pUETL) and defined it as the clear toxic dose giving similar severity for both newborn and young rats (at the same endpoints as far as possible). However, this was not simple to apply because the toxicity profile differed from chemical to chemical and also from newborn to young rats, the number per dose setting usually only being three in each group. Therefore, the most practical analytical strategy had to be a case by case approach. In most cases, the appropriate pUETL for either newborn or young rats was chosen first, thereafter the matching toxic dose or the range of doses was estimated giving similar severity for either group of rats, considering the whole data balance. Again, data from the dose-finding studies were also taken into account, especially considering the kinds of toxicity which appeared and the limits to be used. It should be noted that pUETL is not an absolute value, being different from pNOAEL/BMDL, but useful nevertheless to compare toxic responses between newborn and young rats at sufficient exposure.

This unique approach using two original definitions, with additional data from dose-finding studies concerning limitations, was fully supported by peer-reviewers of toxicology journals. On the other hand, the BMD approach for our whole data, including the dose-finding studies, was first conducted for this article.

4. Comparison of sensitivity of newborn and young rats to chemicals

4.1. Toxicity profiles

Critical toxicity data and the preliminary evaluation for 18 chemical studies have already been published in the literature. Table 1 shows a summary of the major findings for toxicities from the newborn and young studies. Fourteen chemicals commonly induced similar types of toxicities in both ages with the data considered in the pNOAEL or BMD approaches. With 3-ethylphenol and 1,1,2,2-tetrabromoethane, the toxicity profiles of both ages were not similar. In the case of 3-ethylphenol, the toxic similarity or difference between newborn and young rats cannot be predicted because of inadequate high dose setting in the newborn study. For 1,1,2,2-tetrabromoethane, hepatotoxicity in newborn rats can be speculated to appear at higher doses because a remarkable increase of relative liver weight was observed in the dose-finding study, although pathological examination was not conducted. In contrast, 2,4,6-trinitrophenol demonstrated a completely different profile of the major toxicities between the differently aged rats; also, tetrabromobisphenol A demonstrated unique toxicity in newborn rats.

4.1.1. Specific toxicity to reproductive organs in newborn rats

Although specific developmental parameters such as preputial separation and vaginal opening were carefully examined in newborn studies, no significant changes for any chemicals were observed.

In the case of *p*-(α,α-dimethylbenzyl) phenol, ovary weights were lowered at the end of the dosing as well as the recovery-maintenance periods and increased numbers

Table 1
Major types or symptoms of toxicities of 18 industrial chemicals in newborn and young studies

Chemical name	Newborn studies	Young studies	References
4-Nitrophenol	Convulsions	Hypoactivity, convulsions	Koizumi et al. (2001)
2,4-Dinitrophenol	Hypoactivity, convulsions	Hypoactivity, convulsions	Koizumi et al. (2001)
3-Aminophenol	Tremors, thyroid hypertrophy	Tremors, thyroid hypertrophy, anemia	Koizumi et al. (2002)
2-Chlorophenol	Tremors, renal toxicity	Tremors, hypoactivity	Hasegawa et al. (2005)
4-Chlorophenol	Tremors	Tremors, tachypnea	Hasegawa et al. (2005)
2-*tert*-Butylphenol	Hypoactivity, ataxia	Hypoactivity, ataxia	Hirata-Koizumi et al. (2005b)
2,4-Di-*tert*-butylphenol	Hepatic and renal toxicity	Hepatic and renal toxicity	Hirata-Koizumi et al. (2005b)
3-Methylphenol	Tremors, hyperactivity	Tremors	Koizumi et al. (2003)
3-Ethylphenol	Low BW	Ataxia, forestomach lesions	Takahashi et al. (2006)
4-Ethylphenol	Hypoactivity, delayed reflexes	Ataxia, forestomach lesions	Takahashi et al. (2006)
p-(α,α-Dimethylbenzyl) phenol	Renal toxicity, ovarian lesions	Renal toxicity, forestomach lesions	Hasegawa et al. (2005)
1,3,5-Trihydroxybenzene	Thyroid hypertrophy	Thyroid hypertrophy	Hasegawa et al. (2005)
2,4,6-Trinitrophenol	Low BW	Anemia, testicular atrophy	Takahashi et al. (2004)
(Hydroxyphenyl)methyl phenol	Low BW	Low BW, forestomach lesions	Hasegawa et al. (2005)
Trityl chloride	Low BW, hepatotoxicity	Low BW, hepatotoxicity	Hasegawa et al. (2005)
1,3-Dibromopropane	Low BW, hepatotoxicity	Low BW, hepatotoxicity, anemia	Hirata-Koizumi et al. (2005a)
1,1,2,2-Tetrabromoethane	Low BW	Hepatotoxicity	Hirata-Koizumi et al. (2005a)
Tetrabromobisphenol A	Renal toxicity	None	Fukuda et al. (2004)

BW: body weight.

of atretic follicles at the end of the dosing period. Most females continued to show various changes after the recovery-maintenance period, such as decreased numbers of corpa lutea in the ovaries, and hypertrophy of endometrial epithelium in the uteri. Therefore, further studies on this chemical should be conducted to elucidate the underlying mechanisms.

With (hydroxyphenyl)methyl phenol, some estrogenic effects were expected because it consists of bisphenol D, E and F isomers, and bisphenol F is reported to have estrogenic potential on the evidence of several *in vitro* and *in vivo* experiments (Hashimoto et al., 2001; Yamasaki et al., 2002; Stroheker et al., 2003). Some phenols such as nonylphenol, *p-tert*-octylphenol, bisphenol A and diethylstilbestrol have already been reported to induce morphological alteration of sex organs on early phase exposure after birth although the administration routes were either intraperitoneal or subcutaneous (Lee, 1998; Katsuda et al., 2000; Khan et al., 1998; Suzuki et al., 2002). The negative result in our study may be related to an insufficient component level of bisphenol F to induce such action.

4.1.2. Other specific toxicity in newborn rats

There was one exceptional case of toxicity limited to newborn rats. Tetrabromobisphenol A induced polycystic kidneys at 200 and 600 mg/kg in newborn rats but not in doses up to 1000 mg/kg in the main young study and 6000 mg/kg for 18 days exposure in an additional young study. Such specific renal toxicity in newborn rats has also been described for other chemicals such as chlorambucil (Kavlock et al., 1987), tetrachloro-1,4-dibenzodioxine (Couture-Haws et al., 1991) and difluoromethylornithine (Gray and Kavlock, 1991). Kidney nephrons of rats are formed in the period of the advanced stage of pregnancy until 2 weeks after birth (Chevalier, 1998), only 10% of nephrons are present at birth (Merlet-Benichou et al., 1994). It is possible that developing renal tubules in newborn rats may be sensitive to induction of hyperplasia of the tubular epithelium in response to cellular damage, leading to polycystic lesions. Although this toxicity is unusual—at least in newborn rats—it seems reasonable to consider similar unusual potential effects in newborn humans for some chemicals.

4.1.3. Specific toxicity in young rats

2,4,6-Trinitrophenol induced anemia and atrophy of seminiferous tubules of testes in young rats but only slight lowering of body weights in the main newborn study. Higher doses in the dose-finding newborn study induced severe suppression of body weight gain and death but not anemia or testicular toxicity. Sertoli cells in rats proliferate rapidly from day 19 of gestation to postnatal Day 15, then slow down and cease multiplying by approximately postnatal Day 20 (Orth, 1982, 1984; Toppari et al., 1996); 2,4,6-trinitrophenol seems unlikely to affect this stage rather affecting the maturation of spermatids. For anemia, the same pattern, of anemia only in young rats, was found for 3-aminophenol and 1,3-dibromopropane. Although methemoglobin levels were not determined in this study, it was reported that methemoglobin reductase levels in newborn rats are distinctly higher than in young animals (Gruener, 1976; Lo and Agar, 1986), which could be a reason for higher susceptibility in the latter. Another possible explanation is that major metabolites such as picramic acid may damage seminiferous tubules as well as induce hemolytic anemia but the metabolic rate may be very slow in newborn rats because of low P450 content (Rich and Boobis, 1997).

Hyperplasia of squamous cells in forestomach was observed for 3-ethylphenol, 4-ethylphenol, *p*-(α,α-dimethylbenzyl) phenol and (hydroxyphenyl)methyl phenol only at high toxic doses in young rats. Generally, phenols have similar toxicological effects due to their actions as extremely corrosive protoplasmic poisons (Manahan, 2003; Bloom and Brandt, 2001). The fact that the epithelium of the gastrointestinal tract of newborn rats may be more quickly renewed than that of young rats because of more active body metabolism in developing newborn rats, as well as a low capacity for gastric acid secretion, could explain any lower sensitivity in this regard.

4.2. Comparison of pNOAELs and pUETLs

pNOAELs for newborn and young rats with all chemicals were re-evaluated as shown in Table 2. Single pNOAELs for newborn and young rats were estimated for most chemicals on the basis of careful analyses of the results from the dose-finding and main studies. In two cases we judged that specification of a single value was not appropriate and therefore ranges were adopted. In case of 3-methylphenol for newborn rats, tremors only with contact stimuli were noted in three males on single days at the medium dose of 100 mg/kg in the main study. Thus the overt NOAEL became the low dose of 30 mg/kg, but the realistic NOAEL was considered to be slightly lower than 100 mg/kg, supported by overt NOAEL at 100 mg/kg in the dose-finding study. Therefore, the pNOAEL was established in the range of 60–80 mg/kg/day for more accurate comparison with data from the young study. The second case concerned the value for 2,4,6-trinitrophenol for newborn rats because they showed only a slight lowering of the body weight at 61.5 mg/kg and the low dose of 16.1 mg/kg was not considered appropriate as the pNOAEL; we adopted the range of 40–50 mg/kg/day instead. It should be noted that the pNOAEL of 1000 mg/kg/day of young rats for tetrabromobisphenol A is also not realistically appropriate because it was the highest limit dose indicated in the Test Guideline. As for estimation of pUETL, 8 values were given as ranges based on the definition of matching toxic dose ranges to induce clear toxicity at similar severity as described earlier. There were two chemicals without matches: 3-ethylphenol and tetrabromobisphenol A. For the former case, a dose in

Table 2
Summary of pNOAELs and pUETLs for 18 industrial chemicals in newborn and young rats

Chemical name	Newborn studies		Young studies		Young/Newborn	
	pNOAEL	pUETL	pNOAEL	pUETL	pNOAEL	pUETL
	(mg/kg/day)		(mg/kg/day)			
4-Nitrophenol	110	230	400	600–800	3.6	2.6–3.5
2,4-Dinitrophenol	10	30	20	80	2.0	2.7
3-Aminophenol	80	240	240	720	3.0	3.0
2-Chlorophenol	40	200–250	200	1000	5.0	4.0–5.0
4-Chlorophenol	100	300	100	500	1.0	1.7
2-*tert*-Butylphenol	20	100–150	100	500	5.0	3.3–5.0
2,4-Di-*tert*-butylphenol	5	100	20	500	4.0	5.0
3-Methylphenol	60–80	300	300	1000	4.0–5.0	3.3
3-Ethylphenol	100	—	300	—	3.0	—
4-Ethylphenol	30	200–250	100	1000	3.3	4.0–5.0
p-(α,α-Dimethylbenzyl) phenol	30	300	100	700–800	3.3	2.3-2.7
1,3,5-Trihydroxybenzene	100	500	300	1000	3.0	2.0
2,4,6-Trinitrophenol	40–50	65	20	100	0.4–0.5	1.5
(Hydroxyphenyl)methyl phenol	100	140–160	40	1000	0.4	6.3–7.1
Trityl chloride	60	400–500	12	300	0.2	0.6–0.8
1,3-Dibromopropane	50	150	10	250	0.2	1.7
1,1,2,2-Tetrabromoethane	50	200	6	300–400[a]	0.1	1.5–2.0
Tetrabromobisphenol A	40	—	1000[b]	—	25[b]	—

—: Appropriate values were not able to be given.
[a] These range values were estimated on the basis of all relevant toxicity data, including single dose toxicity data in young rats (the lowest mortality dose was 722 mg/kg for males and 852 for females) (MHLW, 2003).
[b] No accurate values for pNOAEL and pNOAEL ratio could be generated because 1000 mg/kg/day is the highest dose indicated in the Test Guideline.

newborn rats matching the toxic dose in young rats could not be predicted because the high dose in newborn rats did not induce any clear toxicity. The latter is that the high dose in young rats did not induce any toxicity.

The last column in Table 2 shows ratios for the young/newborn pNOAELs, and young/newborn pUETLs. Among the pNOAEL ratios for all 18 chemicals, newborn rats were less or nearly equal in sensitivity (less than 2-fold) to 6 chemicals (33%), clearly more sensitive (2–5-fold) to 11 chemicals (61%) and more than 25-fold for one exceptional case (6%). The mean ratio was 3.9 for all 18 chemicals or 2.5 for all but the exceptional case. Among the available pUETL ratios for 16 chemicals, 5 were less or nearly equal in newborn rats (less than 2-fold change) (31%) and 11 chemicals were clearly more toxic (2–8-fold) (69%). The mean ratio was 3.1 for the 16 chemicals.

Based on reliable calculated ratios for our two endpoints, approximately 94% of values (32 out of 34 ratios) demonstrated differences of 5-fold or less, one chemical had a 6–8-fold variation, and in the case of a 25-fold ratio of tetrabromobisphenol A, the nephrotoxicity in newborn rats is a specific toxicity rather than a higher susceptibility to the same toxic endpoint in young rats. These same ratios can be used to state that a higher susceptibility (more than 2-fold) in newborn rats was found for 62% of all tested chemicals in terms of pNOAELs and pUETLs, via oral repeated administration.

To appraise correlations between pUETL and pNOAEL ratios (young/newborn rats), available values were plotted on a logarithmic scale in a correlation diagram. As shown in Fig. 2, two separate groups became apparent, group 1 has the same or lower pNOAELs for newborn than young rats, and group 2 has higher pNOAELs for newborn than young rats. The mechanistic speculation for the differences is discussed next.

4.3. Speculation on differences in responses between low and high doses in newborn and young rats

Immature functions of organs (especially the liver and kidneys), in newborn rats may contribute to the difference of response. There were at least two types of dose response curve shifts between newborn and young rats, as illustrated in Fig. 3. The first was a parallel shift from right (young) to left (newborn) for 12 phenolic chemicals (group 1). The other 5 chemicals demonstrated a steeper shaped curve in newborn than young rats but young rats were clearly more sensitive around the pNOAEL doses (group 2).

Group 1 chemicals may primarily have direct actions on their target organs such as the central nervous system, kidneys or thyroid. They may be detoxified by the formation of conjugates, for example, glucuronidation of 4-nitrophenol (Robinson et al., 1951) and 3-methylphenol (Bray et al., 1950). UDP-glucuronyltransferase activity at birth in the rat liver is known to be comparable to that in adults but nearly 50% lower during nursing (Watkins and Klaassen, 1985; Rachmel and Hazelton, 1986). Therefore, a low capacity for glucuronidation may be one of the major causes of higher susceptibility of newborn rats to these phenols. This may also occur in human infants since immature hepatic glucuronidation and low activity of bilirubin glucuronidation at birth have been shown in human infants

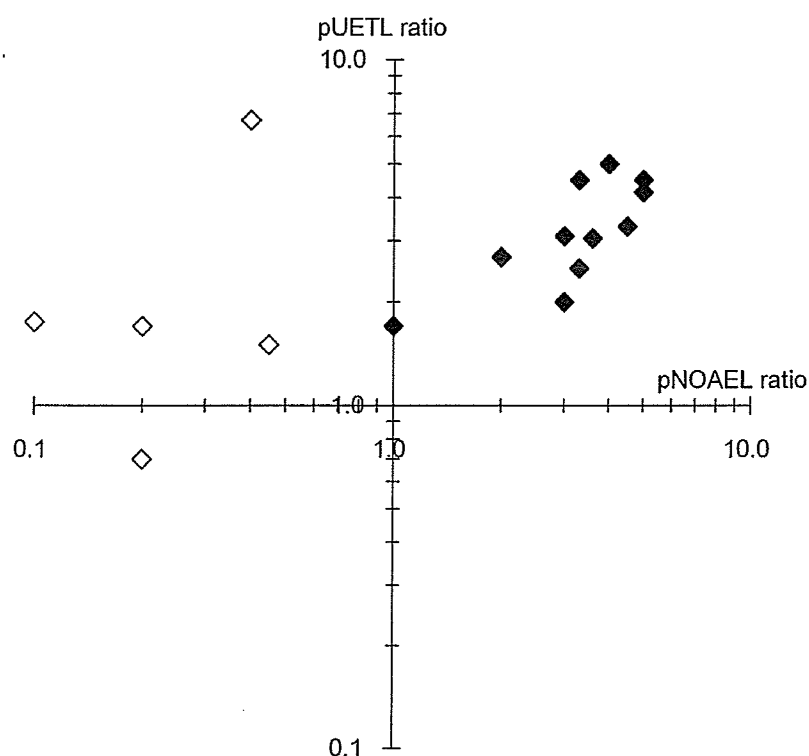

Fig. 2. Correlations of pUETL and pNOAEL ratios (young/newborn). Each point is plotted on a logarithmic scale from the ratios for young/newborn pUETLs and pNOAELs. Closed and open diamonds indicate group 1 and 2 chemicals, respectively.

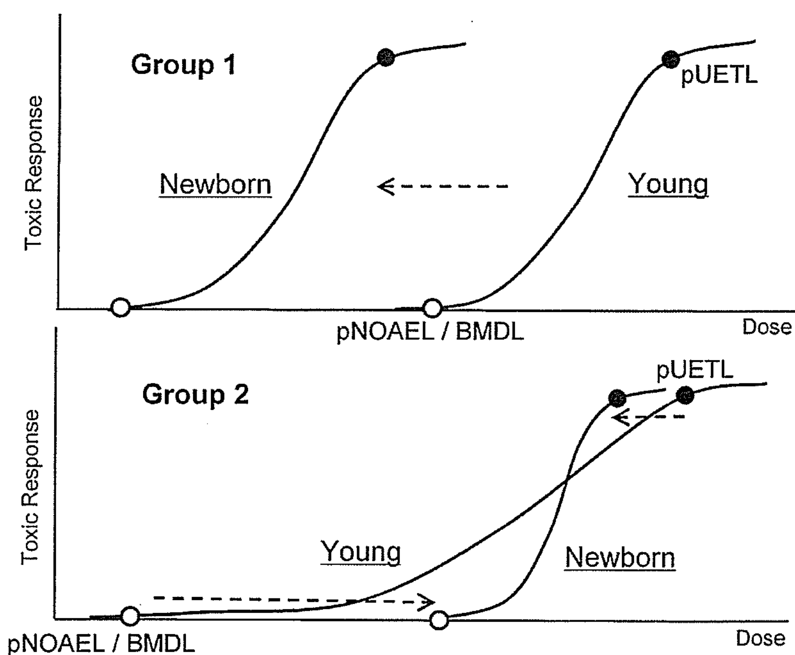

Fig. 3. Illustration of two patterns of shift of dose response curves from young to newborn rats.

(Gow et al., 2001; Kawade and Onishi, 1981). In addition, there is a possibility that high susceptibility may be due to a low capacity for hepatic cytochrome P450 (Rich and Boobis, 1997) and renal excretion (Horster, 1977), and increased permeability of the blood-brain barrier (Cremer et al., 1979).

Group 2 chemicals did not demonstrate as many effects at the low dose but nearly the same or higher

number of effects at the high dose in newborn compared to young rats. These chemicals may need metabolic activation to exert toxic effects. Newborn rats have been shown to have a low content of hepatic cytochrome P450 (Rich and Boobis, 1997) and a drop of glutathione-S-transferase activity in the early days after birth (Tee et al., 1992). Therefore, production of active metabolites may be significantly lower in newborn rats. In fact, it has been suggested that 1,1,2,2-tetrabromoethane requires an oxidative biotransformation to produce active intermediates (Kennedy et al., 1993) and 1,3-dibromopropane is conjugated with glutathione before or after oxidative biotransformation (James et al., 1981) as is common for dihaloalkanes or dihaloalkenes (Zoetemelk et al., 1986; Trevisan et al., 1989). However, pUETLs for newborn rats for 4–5 chemicals were in approximately the same ranges as in young rats. Although major reasons for variation in susceptibility are unclear, one possible explanation might be a low capacity for protection against deleterious oxidative stress in the newborn when the toxic chemical burden crosses a threshold in the liver, which has a low activity of catalase and glutathione peroxidase during the nursing period (Yoshida et al., 1982).

4.4. Application trial of the BMD approach

We attempted to derive BMDLs as sensitive and appropriate endpoints in each study in addition to pNOAELs whenever possible. These calculated values are shown in Table 3. Most BMDLs seem to be relatively close to the corresponding pNOAELs but there are some cases in which BMDLs were lower than the probable values from toxicity profiles. One major reason may be the nature of the toxicity data used for the BMDL calculations. For example, no changes were observed with histopathological data in the young study for trityl chloride at 12 mg/kg, only slight changes in 3 of 6 animals at 60 mg/kg, and 4 mild and 2 moderate levels of change in 6 animals at 300 mg/kg. For the BMDL estimation from these data we input an incidence of 3 animals in 6 at 60 mg/kg and 6 animals in 6 animals at 300 mg/kg, even though the severities of these changes were different. So an actual dose response curve was obviously steeper than the input data curve, leading to a lower BMDL of 6.8 mg/kg/day, compared to the pNOAEL of 12 mg/kg/day. Nonetheless, Fig. 4 shows a good relationship between pNOAEL and BMDL since the correlation efficient was 0.904 (calculated without logarithmic conversion). The BMDL ratios in Table 3 are slightly lower than pNOAEL ratios in Table 2, with 9 chemicals (53%) demonstrating less or nearly equal sensitivity in newborn rats (less than 2-fold) and 8 chemicals (47%) demonstrating more sensitivity (2–8-fold) in newborn rats. However, a correlation diagram of the pUETL ratios versus the BMDL ratios also showed the same profile as Fig. 2 (not shown here). Therefore, the BMD approach can be considered very useful for the present purposes and somewhat easier than our pNOAEL estimation because extensive experience in toxicology is necessary for the latter estimations.

Table 3
Summary of BMDL values and ratios for 18 industrial chemicals in newborn and young rats

Chemical name	Newborn studies (mg/kg/day)	Young studies (mg/kg/day)	Young/newborn
4-Nitrophenol	141	392	2.8
2,4-Dinitrophenol	11	14	1.3
3-Aminophenol	54	254	4.7
2-Chlorophenol	31	126	4.1
4-Chlorophenol	79	63	0.8
2-*tert*-Butylphenol	43	130	3.0
2,4-Di-*tert*-butylphenol	7.5	48	5.1
3-Methylphenol	50	397	7.9
3-Ethylphenol	276	376	1.4
4-Ethylphenol	53	173	3.3
p-(α,α-Dimethylbenzyl) phenol	28	42	1.5
1,3,5-Trihydroxybenzene	63	206	3.3
2,4,6-Trinitrophenol	41	15	0.4
(Hydroxyphenyl)methyl phenol	108	42	0.4
Trityl chloride	34	6.8	0.2
1,3-Dibromopropane	32	6.1	0.2
1,1,2,2-Tetrabromoethane	82	3.1	0.04
Tetrabromobisphenol A	45	—	—

—, Appropriate values could not be generated because no toxicity was apparent in the young study.

5. Discussion of pediatric susceptibility

Major uncertainty exists in the derivation of human safety doses from animal experimental data. This uncertainty consists primarily of toxicokinetic and toxicodynamic differences between experimental animals and humans and among humans, and is addressed through the use of two factors, inter-species differences and human variability (intra-species differences). For either factor, a value of 10-fold has generally been applied for most assessments.

The aim of risk assessment is to derive the estimated no adverse toxic response level in sensitive humans. Thus, NOAELs or BMDLs are used as the starting point values, and not higher doses exhibiting toxicity, although descriptions of such toxicity provide critical information on risk assessment. Human variability implies appreciable differences of NOAELs or BMDLs between average populations and sensitive subpopulations as indicated by Dourson et al. (2002). Since the general human population or a more uniform experimental animal population is typically the focus group for toxicity evaluation, risk assessment needs to include sensitive subpopulations, such as infants, children, the elderly, and specific subgroups with minor diseases or relevant genetic polymorphisms. However, some hypersusceptible individuals might be excluded, for example, patients with severe hepatic or renal dysfunction should