received TPTs during pregnancy at doses that did not cause overt maternal toxicity. In a rat two-generation reproductive toxicity study, TBTCl at relatively low doses affected male and female reproductive systems, including decreased weights of the male reproductive organs, decreased counts of spermatids and sperms, decrease in serum estradiol levels, delayed vaginal opening, impaired estrous cyclicity, and increased female AGD. TBTCl and DBTCl during early pregnancy caused implantation failure in rats. Implantation failure due to TBTCl and DBTCl, at lower doses than TBTCl, may be mediated via the suppression of uterine decidualization and correlated with the reduction in serum progesterone levels. Administration of MBTCl during early pregnancy did not cause pre- or postimplantation loss. Maternal exposure during pregnancy to TBTs caused embryonic/fetal deaths, suppression of fetal growth, and cleft palate at maternal toxic doses. Significant effects on growth profiles and decreased liver weights were reported in offspring of rats given TBTCl by gavage, even at 0.025 mg/kg from day 8 of pregnancy until adulthood. Behavioral changes were also shown in postnatal offspring of rats that received TBTs during pregnancy at doses that did not cause overt maternal toxicity. Many reports demonstrated that DBT derivatives with different anions, such as dichloride, diacetate, maleate, dilaurate, and oxide, are teratogenic when administered during organogenesis in rats. Rat embryos are the most susceptible to teratogenic effects of DBT on day 8 of pregnancy after maternal exposure. The developmental toxicity studies on butyltins suggest that the teratogenic effects of DBT are different from those of TeBT, TBT, and MBT in its mode of action. DBTCl exerts dysmorphogenic effects on postimplantation embryos *in vitro*. The phase specificity for the in vivo teratogenic effects of DBTCl may be attributable to a decline in the susceptibility of embryos to the dysmorphogenesis of DBTCl with advancing development. The findings of *in vivo* and *in vitro* studies suggest that DBT itself is a causative agent in DBT teratogenesis. Because the teratogenicity of DTB has been reported in a single species, studies in additional species would be of great value in evaluating developmental toxicity of DBT. As for miscellaneous organotin compounds, several reports on developmental toxicity are published. Prenatal and/or postnatal exposure to TMTCl or THTCl caused behavioral changes in postnatal rat offspring. Behavioral changes in postnatal pups of rats given organotin prenatally and/or postnatally may be a sensitive parameter for reproductive and developmental toxicity. A mixture of DOTTG and MOTTG is developmentally toxic and produces fetal malformations in mice. An increased number of cleft palates was reported in fetuses of rats given DMTCl during organogenesis at severely maternal toxic dose.

References

Adeeko, A., Li, D., Forsyth, D.S., Casey, V., Cooke, G.M., Barthelemy, J., Cyr, D.G., Trasler, J.M., Robaire, B., and Hales, B.F. (2003) Effects of in utero tributyltin chloride exposure in the rat on pregnancy outcome, *Toxicological Sciences*, 74, 407–15.

Baroncelli, S., Karrer, D., and Turillazzi, P.G. (1990) Embryotoxic evaluation of bis(tri-*n*-butyltin)oxide (TBTO) in mice, *Toxicology Letters*, 50, 257–62.

Baroncelli, S., Karrer, D., and Turillazzi, P.G. (1995) Oral bis(tri-*n*-butyltin) oxide in pregnant mice. I. Potential influence of maternal behavior on postnatal mortality, *Journal of Toxicology and Environmental Health*, 46, 355–67.

Belfoid, A.C., Purperhart, M., and Ariese, F. (2000) Organotin levels in seafood, *Marine Pollution Bulletin*, 40, 226–32.

Bettin, C., Oehlmann, J., and Stroben, E. (1996) YBY-induced imposex in marine neogastropods is mediated by an increasing androgen level, *Helgoländer Meeresunters*, 50, 299–317.

Boyer, I.J. (1989) Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals, *Toxicology*, 55, 253–98.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G., and Burt, G.R. (1986) The decline of the gastropod *Nucella lapillus* around South-West England: evidence for the effect of tributyltin from antifouling paints, *Journal of the Marine Biological Association of the United Kingdom*, 66, 611–40.

Bryan, G.W., Gibbs, P.E., Burt, G.R., and Hummerstone, L.G. (1987) The effect of tributyltin (TBT) accumulation on adult dog-whelks, *Nucella lapillus*: long-term field and laboratory experiments, *Journal of the Marine Biological Association of the United Kingdom*, 67, 525–44.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G., and Burt. G.R. (1988) Comparison of the effectiveness of tri-*n*-butyltin chloride and five other organotin compounds in promoting the development of imposex in the dogwhelk *Nucella lapillus*, *Journal of the Marine Biological Association of the United Kingdom*, 68, 733–44.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G., and Burt, G.R. (1989) Uptake and transformation of 14-C labelled tributyltin chloride by the dog-whelk, *Nucella lapillus*: Importance of absorption from the diet, *Marine Environmental Research*, 28, 241–5.

Bui, Q.Q., Tran, M.B., and West, W.L. (1986) A comparative study of the reproductive effects of methadone and benzo[*a*]pyrene in the pregnant and pseudopregnant rat, *Toxicology*, 42, 195–204.

Chernoff, N., Setzer, R.W., Miller, D.B., Rosen, M.B., and Rogers, J.M. (1990) Effects of chemically induced maternal toxicity on prenatal development in the rat, *Teratology*, 42, 651–8.

Clark, R.L., Anderson, C.A., Prahalada, S., Robertson, R.T., Lochry, E.A., Leonard, Y.M., Stevens, J.L., and Hoberman, A.M. (1993) Critical developmental periods for effects on male rat genitalia induced by finasteride, a 5 alpha-reductase inhibitor, *Toxicology and Applied Pharmacology*, 119, 34–40.

Colborn, T., vom Saal, F., and Soto, A. (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans, *Environmental Health Perspectives*, 101, 378–84.

Cooke, G.M., Tryphonas, H., Pulido, O., Caldwell, D., Bondy, G.S., and Forsyth, D. (2004) Oral (gavage), in utero and postnatal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part I: toxicology, histopathology and clinical chemistry, *Food and Chemical Toxicology*, 42, 211–20.

Crofton, K.M., Dean, K.F., Boncek, V.M., Rosen, M.B., Sheets, L.P., Chernoff, N., and Reiter, L.W. (1989) Prenatal or postnatal exposure to bis(tri-*n*-butyltin)oxide in the rat: postnatal evaluation of teratology and behavior, *Toxicology and Applied Pharmacology*, 97, 113–23.

Cummings, A.M. (1990) Toxicological mechanisms of implantation failure, *Fundamental and Applied Toxicology*, 15, 571–9.

Davies, J.M., Bailey, S.K., and Moore, D.C. (1987) Tributyltin in Scottish sea lochs, as indicated by degree of imposex in the dogwhelk, *Nucella lapillus* (L.), *Marine Pollution Bulletin*, 18, 400–4.

Davis, A., Barale, R., Brun, G., Forster, R., Güunther, T., Hautefeuille, H., van der Heijden, C.A., Knaap, A.G.A.C., Krowke, R., Kuroki, T., Loprieno, N., Malaveille, C., Merker, H.J., Monaco, M., Mosesso, P., Nuebert, D., Norppa, H., Sorsa, M., Vogel, E., Voogd, C.E., Umeda, M., and Bartsch, H. (1987) Evaluation of the genetic and embryotoxic effects of bis(tri-*n*-butyltin)oxide (TBTO), a broad-spectrum pesticide, in multiple in vivo and in vitro short-term tests, *Mutation Research*, 188, 65–95.

De Feo, V.J. (1963) Temporal aspect of uterine sensitivity in the pseudopregnant or pregnant rat, *Endocrinology*, 72, 305–16.

Doering, D.D., Stechelbroeck, S., Doering, T., and Klingm, D. (2002) Effects of butyltins on human 5-reductase type 1 and type 2 activity, *Steroids*, 67, 859–67.

Eisler, R. (2000) Tin, in *Handbook of Chemical Risk Assessment: Health Hazards to Humans, Plants, and Animals, Volume 1 (Metals)*, Boca Raton, FL, Lewis Publishers, 551–603.

Ema, M. and Harazono, A. (2000) Adverse effects of dibutyltin dichloride on initiation and maintenance of rat pregnancy, *Reproductive Toxicology*, 14, 451–6.

Ema, M. and Harazono, A. (2001) Toxic effects of butyltin trichloride during early pregnancy in rats, *Toxicology Letters*, 125, 99–106.

Ema, M. and Miyawaki, E. (2001) Role of progesterone on suppression of uterine decidualization and implantation failure induced by triphenyltin chloride in rats, *Congenital Anomalies*, 41, 106–11.

Ema, M. and Miyawaki, E. (2002) Suppression of uterine decidualization correlated with reduction in serum progesterone levels as a cause of preimplantation embryonic loss induced by diphenyltin in rats, *Reproductive Toxicology*, 16, 309–17.

Ema, M., Itami, T., and Kawasaki, H. (1991) Teratogenicity of di-*n*-butyltin dichloride in rats, *Toxicology Letters*, 58, 347–56.

Ema, M., Itami, T., and Kawasaki, H. (1992) Susceptible period for the teratogenicity of di-*n*-butyltin dichloride in rats, *Toxicology*, 73, 81–92.

Ema, M., Kurosaka, R., Amano, H., and Ogawa, Y. (1995a) Further evaluation of the developmental toxicity of tributyltin chloride in rats, *Toxicology*, 96, 195–201.

Ema, M., Kurosaka, R., Amano, H., and Ogawa, Y. (1995b) Comparative developmental toxicity of butyltin trichloride, dibutyltin dichloride and tributyltin chloride in rats, *Journal of Applied Toxicology*, 15, 297–302.

Ema, M., Iwase, T., Iwase, Y., and Ogawa, Y. (1995c) Dysmorphogenic effects of di-*n*-butyltin dichloride in cultured rat embryos, *Toxicology in Vitro*, 9, 703–9.

Ema, M., Kurosaka, R., Amano, H., and Ogawa, Y. (1996a) Comparative developmental toxicity of di-, tri- and tetrabutyltin compounds after administration during late organogenesis in rats, *Journal of Applied Toxicology*, 16, 71–6.

Ema, M., Iwase, T., Iwase, Y., Ohyama, N., and Ogawa, Y. (1996b) Change of embryotoxic susceptibility to di-*n*-butyltin dichloride in cultured rat embryos, *Archives of Toxicology*, 70, 742–8.

Ema, M., Miyawaki, E., Harazono, A., and Ogawa, Y. (1997a) Effects of triphenyltin chloride on implantation and pregnancy in rats, *Reproductive Toxicology*, 11, 201–6.

Ema, M., Harazono, A., Miyawaki, E., and Ogawa, Y. (1997b) Effect of the day of administration on the developmental toxicity of tributyltin chloride in rats, *Archives of Environmental Contamination and Toxicology*, 33, 90–6.

Ema, M., Miyawaki, E., Harazono, A., and Ogawa, Y. (1998) Reproductive effects of butyl benzyl phthalate in pregnant and pseudopregnant rats, *Reproductive Toxicology*, 12, 127–32.

Ema, M., Miyawaki, E., and Kawashima, K. (1999a) Suppression of uterine decidualization as a cause of implantation failure induced by triphenyltin chloride in rats, *Archives of Toxicology*, 73, 175–9.

Ema, M., Miyawaki, E., and Kawashima, K. (1999b) Adverse effects of diphenyltin dichloride on initiation and maintenance of pregnancy in rats, *Toxicology Letters*, 108, 17–25.

Ema, M., Miyawaki, E., and Kawashima, K. (1999c) Developmental toxicity of triphenyltin chloride after administration on three consecutive days during organogenesis in rats, *Bulletin of Environmental Contamination and Toxicology*, 62, 363–70.

Ema, M., Miyawaki, E., and Kawashima, K. (2000) Critical period for adverse effects on development of reproductive system in male offspring of rats given di-*n*-butyl phthalate during late pregnancy, *Toxicology Letters*, 111, 271–8.

Ema, M., Harazono, A., Hirose, A., and Kamata, E. (2003) Protective effects of progesterone on implantation failure induced by dibutyltin dichloride in rats, *Toxicology Letters*, 143, 233–8.

Epstein, S.S., Arnold, E., Andrea, J., Bass, W., and Bishop, Y. (1972) Detection of chemical mutagens by the dominant lethal assay in the mouse, *Toxicology and Applied Pharmacology*, 23, 288–325.

Evans, D.W. and Laughlin, R.B., Jr. (1984) Accumulation of bis(tributyltin) oxide by the mud crab, *Rhithropanopeus harrisii*, *Chemosphere*, 13, 213–9.

Faqi, A.S., Schweinfurth, H., and Chahoud, I. (1997) Determination of the no-effect dose of bis(tri-*n*-butyltin)oxide (TBTO) for maternal toxicity and teratogenicity in mice, *Congenital Anomalies*, 37, 251–8.

Faqi, A.S., Schweinfurth, H., and Chahoud, I. (2001) Developmental toxicity of an octyltin stabilizer in NMRI mice, *Reproductive Toxicology*, 15, 117–22.

Farr, C.H., Reinisch, K., Holson, J.F., and Neubert, D. (2001) Potential teratogenicity of di-*n*-butyltin dichloride and other dibutyltin compounds, *Teratogenesis, Carcinogenesis, and Mutagenesis*, 21, 405–15.

Fent, K. (1996) Ecotoxicology of organotin compounds, *Critical Reviews in Toxicology*, 26, 1–117.

Fent, K. and Hunn, J. (1991) Phenyltins in water, sediment, and biota of freshwater marinas, *Environmental Science and Technology*, 25, 956–63.

Fent, K. and Meier, W. (1992) Tributyltin-induced effects on early life stages of minnows *Phoxinus phoxinus*, *Archives of Environmental Contamination and Toxicology*, 22, 428–31.

Fent, K. and Meier, W. (1994) Effects of triphenyltin on fish early life stages, *Archives of Environmental Contamination and Toxicology*, 27, 224–31.

Féral, C. and LeGall, S. (1983) The influence of a pollutant factor (tributyltin) on the neuroendocrine mechanism responsible for the occurrence of a penis in the female of *Ocenebra erinacea*, in *Molluscan neuro-endocrinology*, Lever, J. and Boer, H., Eds., Amsterdam: North Holland Publishing Co., 173–5.

Fioroni, P., Oehlmann, J., and Stroben, E. (1991) The pseudohermaphroditism of prosobranchs; morphological aspects, *Zoologischer Anzeiger*, 226, 1–26.

Fish, R.H., Kimmel, E.C., and Casida, J.E. (1976) Bioorganotin chemistry: reactions of tributyltin derivatives with a cytochrome P-450 dependent monooxygenese enzyme system, *Journal of Organometallic Chemistry*, 118, 41–54.

Gaines, T.B. and Kimbrough, R.D. (1968) Toxicity of fentin hydroxide to rats, *Toxicology and Applied Pharmacology*, 12, 397–403.

Gårdlund, A.T., Archer, T., Danielsson, B., Fredriksson, A., Lindqvist, N.G., Lindstrom, H., and Luthman, J. (1991) Effects of prenatal exposure to tributyltin and trihexyltin on behaviour in rats, *Neurotoxicology and Teratology*, 13, 99–105.

Giavini, E., Prati, M., and Vismara, C. (1980) Effects of triphenyltin acetate on pregnancy in the rat, *Bulletin of Environmental Contamination and Toxicology*, 24, 936–9.

Gibbs, P.E. and Bryan, G.W. (1986) Reproductive failure in populations of the dog-welk, *Nucella lapillus*, caused by imposex induced by tributyltin from antifouling paints, *Journal of the Marine Biological Association of the United Kingdom*, 66, 767–77.

Gibbs, P.E., Bryan, G.W., Pascoe, P.L., and Burt, G.R. (1987) The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination, *Journal of the Marine Biological Association of the United Kingdom*, 67, 507–23.

Gibbs, P.E., Pascoe, P.L., and Burt, G.R. (1988) Sex change in the female dog-whelk, *Nucella lapillus*, induced by tributyltin from antifouling paints, *Journal of the Marine Biological Association of the United Kingdom*, 68, 715–31.

Harazono, A. and Ema, M. (2000) Suppression of decidual cell response induced by tributyltin chloride in pseudopregnant rats: as a cause of early embryonic loss, *Archives of Toxicology*, 74, 632–7.

Harazono, A. and Ema, M. (2003) Suppression of decidual cell response induced by dibutyltin dichloride in pseudopregnant rats: As a cause of early embryonic loss, *Reproductive Toxicology*, 17, 393–9

Harazono, A., Ema, M., and Kawashima, K. (1998a) Evaluation of malnutrition as a cause of tributyltin-induced pregnancy failure in rats, *Bulletin of Environmental Contamination and Toxicology*, 61, 224–30.

Harazono, A., Ema, M., and Kawashima, K. (1998b) Evaluation of early embryonic loss induced by tributyltin chloride in rats: Phase- and dose-dependent antifertility effects, *Archives of Environmental Contamination and Toxicology*, 34, 94–9.

Harazono, A., Ema, M., and Ogawa, Y. (1996) Pre-implantation embryonic loss induced by tributyltin chloride in rats, *Toxicology Letters*, 89, 185–90.

Haubruge, E., Petit, F., and Gage, M.J. (2000) Reduced sperm counts in guppies (*Poecilia reticulata*) following exposure to low levels of tributyltin and bisphenol A, *Proceedings of the Royal Society of London. Series B. Biological Sciences*, 267, 2333–7.

Hawkins, L.E. and Hutchinson, S. (1990) Physiological and morphogenetic effects of monophenyltin trichloride on *Ocenebra erinacea* (L.), *Functional Ecology*, 4, 449–54.

Holm, G., Norrgren, L., and Linden, O. (1991) Reprodictive and histophathological effects of long-term experiemental exposure to bis(tributyltin) oxide (TBTO) on the three-spined stickeback, *Gasterosteus aculeatus* Linnaeus, *Journal of Fish Biology*, 38, 373–86.

Horiguchi, T., Shiraishi, H., Shibata, Y., Morita, M., and Shimizu, M. (1996) Imposex and organotin contamination in gastropods after the regulation of organotin use in Japan, *Abstract Book of SETAC 17th Annual Meeting*, 177.

Horiguchi, T., Shiraishi, H., Shimizu, M., and Morita, M. (1997a) Imposex in sea snails, caused by organotin (tributyltin and triphenyltin) pollution in Japan: a survey, *Applied Organometallic Chemistry*, 11, 451–5.

Horiguchi, T., Shiraishi, H., Shimizu, M., and Morita, M. (1997b) Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell thais clavigera, *Environmental Pollution*, 95, 85–91.

Horiguchi, T., Kojima, M., Kaya, M., Matsuo, T., Shiraishi, H., Morita, M., and Adachi, Y. (2002) Tributyltin and triphenyltin induce spermatogenesis in ovary of female abalone, Haliotis gigantea, *Marine Environmental Research*, 54, 679–84.

International Programme on Chemical Safety (IPCS) (1999a) *Concise International Chemical Assessment Document, No. 14 Tributyltin Oxide*, IPCS, Geneva, World Health Organization.

International Programme on Chemical Safety (1999b) *Concise International Chemical Assessment Document, No. 13 Triphenyltin Compounds*, Geneva, World Health Organization.

Ishizaka, T., Suzuki, T., and Saito, Y. (1989) Metabolism of dibutyltin dichloride in male rats, *Journal of Agricultural and Food Chemistry*, 37, 1096–101.

Itami, T., Ema, M., Amano, H., Murai, T., and Kawasaki, H. (1990) Teratogenic evaluation of tributyltin chloride in rats following oral exposure, *Drug and Chemical Toxicology*, 13, 283–95.

Iwai, H., Wada, O., and Arakawa, Y. (1981) Determination of tri-, di-, and monobutyltin and inorganic tin in biological materials and some aspects of their metabolism in rats, *Journal of Analytical Toxicology*, 5, 300–6.

Japan Environment Agency (1998) *Strategic Programs on Environmental Endocrine Disruptors '98*, Tokyo, Environmental Health Department.

Kamrin, M.A., Carney, E.W., Chou, K., Cummings, A., Dostal, L.A., Harris, C., Henck, J.W., Loch-Caruso, R., and Miller, R.K. (1994) Female reproductive and developmental toxicology: overview and current approaches, *Toxicology Letters*, 74, 99–119.

Kannan, K., Tanabe, S., and Tatsukawa, R. (1995) Phenyltin residues in horseshoe crabs, *Tachypleus tridentatus* from Japanese coastal waters, *Chemosphere*, 30, 925–32.

Kannan, K., Corsolini, S., Focardi, S., Tanabe, S., and Tatsukawa, R. (1996) Accumulation pattern of butyltin compounds in dolphin, tuna, and shark collected from Italian coastal waters, *Archives of Environmental Contamination and Toxicology*, 31, 19–23.

Karrer, D., Baroncelli, S., and Turillazzi, P.G. (1995) Oral bis(tri-*n*-butyltin) oxide in pregnant mice. II. Alterations in hematological parameters, *Journal of Toxicology and Environmental Health*, 46, 369–77.

Kenaga, E.E. (1965) Triphenyl tin compounds as insect reproduction inhibitors, *Journal of Economic Entomology*, 58, 4–8,

Kime, D.E., Huyskens, G., McAllister, B.G., Ruranguwa, E., Skorkowski, G., and Ollevia, F. (2001) Tributyltin disrupts the vertebrate reproductive system at muliple sites, *Abstracts 11th Annual Meeting of SETAC Europe*, Madrid, May 6–10, 195.

Kimmel, E.C., Fish, R.H., and Casida, J.E. (1977) Bioorganotin chemistry. Metabolism of organotin compounds in microsomal monooxygenase system and in mammals, *Journal of Agricultural and Food Chemistry*, 25, 1–9.

Krowke, R., Bluth, U., and Neubert, D. (1986) In vitro studies on the embryotoxic potential of (bis[tri-*n*-butyltin])oxide in a limb bud organ culture system, *Archives of Toxicology*, 58, 125–9.

Kumasaka, K., Miyazawa, M., Fujimaki, T., Tao, H., Ramaswamy, B.R., Nakazawa, H., Makino, T., and Satoh, S. (2002) Toxicity of the tributyltin compound on the testis in premature mice, *Journal of Reproduction and Development*, 48, 591–7.

Lau, M.M. (1991) Tributyltin antifoulings: a threat to the Hong Kong marine environment, *Archives of Environmental Contamination and Toxicology*, 20, 299–304.

Laughlin, R.B., Jr., French, W., and Guard, H.E. (1986) Accumulation of bis(tributyltin) oxide by the marine mussel *Mytilus edulis*, *Environmental Science and Technology*, 20, 884–90.

Lehotzky, K., Szeberenyi, J.M., Gonda, Z., Horkay, F., and Kiss, A. (1982) Effects of prenatal triphenyl-tin exposure on the development of behavior and conditioned learning in rat pups, *Neurobehavioral Toxicology and Teratology*, 4, 247–50.

Maguire, R.J. (1991) Aquatic environmental aspects of non-pesticidal organotin compounds, *Water Pollution Research Journal of Canada*, 26, 243–360.

Manning, C.S., Lytle, T.F., Walker, W.W., and Lytle, J.S. (1999) Life-cycle toxicity of bis(tributyltin) oxide to the sheepshead minnow (*Cyprinodon variegatus*), *Archives of Environmental Contamination and Toxicology*, 37, 258–66.

Miyake, K., Misawa, T., Aikawa, H., Yoshida, T., and Shigeta, S. (1989) The effects of prenatal trimethyltin exposure on development and learning in the rat, *Japanese Journal of Industrial Health*, 31, 363–71 (in Japanese).

Miyake, K., Misawa, T., and Shigeta, S. (1990) Toxicity of bis (tri-*n*-butyltin) oxide on learning and development in the rat, *Nippon Eiseigaku Zasshi*, 45, 926–34 (in Japanese).

Miyake, K., Misawa, T., and Shigeta, S. (1991) The effects of prenatal triphenyltin exposure on learning and development in the rat, *Nippon Eiseigaku Zasshi*, 46, 769–76 (in Japanese).

Newton, D.W. and Hays, R.L. (1968) Histological studies of ovaries in rats treated with hydroxyurea, triphenyltin acetate, and triphenyltin chloride, *Journal of Economic Entomology*, 61, 1668–9.

Nirmala, K., Oshima, Y., Lee, R., Imada, N., Honjo, T., and Kobayashi, K (1999) Transgenerational toxicity of tributyltin and its combined effects with polychlorinated biphenyls on reproductive processes in Japanese medaka (*Oryzias latipes*), *Environmental Toxicology and Chemistry*, 18, 717–21.

Noda, T. (2001) Maternal and fetal toxicity of dimetyltin in rats, *Journal of Health Science*, 47, 544–51.

Noda, T., Morita, S., and Baba, A (1993) Teratogenic effects of various di-*n*-butyltins with different anions and butyl(3-hydroxybutyl)tin dilaurate in rats, *Toxicology*, 85, 149–60.

Noda, T., Morita, S., and Baba, A. (1994) Enhanced teratogenic activity of di-*n*-butyltin diacetate by carbon tetrachloride pretreatment in rats, *Food and Chemical Toxicology*, 32, 321–7.

Noda, T., Morita, S., Shimizu, M., Yamamoto, T., and Yamada, A. (1988) Safety evaluation of chemicals for use in house-hold products (VIII)—teratological studies on dibutyltin diacetate in rats, *Annual Report of Osaka City Institute of Public Health and Environ Sciences*, 50, 66–75 (in Japanese).

Noda, T., Morita, S., Yamano, T., Shimizu, M., and Yamada, A. (1991a) Effects of triphenyltin acetate on pregnancy in rats by oral administration, *Toxicology Letters*, 56, 207–12.

Noda, T., Morita, S., Yamano, T., Shimizu, M., Nakamura, T., Saitoh, M., and Yamada, A. (1991b) Teratogenicity study of tri-*n*-butyltin acetate in rats by oral administration, *Toxicology Letters*, 55, 109–15.

Noda, T., Yamano, T., Shimizu, M., Saitoh, M., Nakamura, T., Yamada, A., and Morita, S. (1992a) Comparative teratogenicity of di-*n*-butyltin diacetate with *n*-butyltin trichloride in rats, *Archives of Environmental Contamination and Toxicology*, 23, 216–22.

Noda, T., Nakamura, T., Shimizu, M., Yamano, T., and Morita, S. (1992b) Critical gestational day of teratogenesis by di-*n*-butyltin diacetate in rats, *Bulletin of Environmental Contamination and Toxicology*, 49, 715–22.

Noda, T., Yamano, T., and Shimizu, M. (2001) Effects of maternal age on teratogenicity of di-*n*-butyltin diacetate in rats, *Toxicology*, 167, 181–9.

Noland, E.A., Taylor, D.H., and Bull, R.J. (1982) Monomethyl-and trimethyltin compounds induce learning deficiencies in young rats, *Neurobehavioral Toxicology and Teratology*, 4, 539–44.

Oberdörster, E. and McClellan-Green, P. (2002) Mechanisms of imposex induction in the mud snail, *Ilyanassa obsoleta*: TBT as a neurotoxin and aromatase inhibitor, *Marine Environmental Research*, 54, 715–8.

Oehlmann, J., Stroben, E., and Fioroni, P. (1991) The morphlogical expression of imposex in *Nucella lapillus* (Linnaeus) (Gastropoda: Muricidae), *Journal of Molluscan Studies*, 57, 375–90.

Oehlmann, J., Stroben, E., Fioroni, P., and Markert, B. (1996) Tributyltin (TBT) effects on *Ocinebrina aciculata* (Gastropoda: Muricidae): imposex development, sterilization, sex change and population decline, *The Science of the Total Environment*, 188, 205–23.

Ogata, R., Omura, M., Shimasaki, Y., Kubo, K., Oshima, Y., Aou, S., and Inoue, N. (2001) Two-generation reproductive toxicity study of tributyltin chloride in female rats, *Journal of Toxicology and Environmental Health A*, 63, 127–44.

Ohhira, S. and Matsui, H. (1993a) Gas chromatographic determination of inorganic tin in rat urine after a single oral administration of stannous chloride and mono-, di-, and triphenyltin chloride, *Journal of Chromatography*, 622, 173–8.

Ohhira, S. and Matsui, H. (1993b) Metabolism of diphenyltin compound in rat liver after a single oral administration of diphenyltin dichloride, *Journal of Agricultural and Food Chemistry*, 41, 607–9.

Omura, M., Ogata, R., Kubo, K., Shimasaki, Y., Aou, S., Oshima, Y., Tanaka, A., Hirata, M., Makita, Y., and Inoue, N. (2001) Two-generation reproductive toxicity study of tributyltin chloride in male rats, *Toxicological Sciences*, 64, 224–32.

Pate, B.D. and Hays, R.L. (1968) Histological studies of testes in rats treated with certain insect chemosterilants, *Journal of Economic Entomology*, 61, 32–4.

Paule, M.G., Reuhl, K., Chen, J.J., Ali, S.F., and Slikker, W., Jr. (1986) Developmental toxicology of trimethyltin in the rat, *Toxicology and Applied Pharmacology*, 84, 412–7.

Piver, WT. (1973) Organotin compounds: industrial applications and biological investigation, *Environmental Health Perspectives*, 4, 61–79.

Quevauviller, P.H., Bruchet, A., and Donard, O.F.X. (1991) Leaching of organotin compounds from poly(vinyl chloride) (PVC) material, *Applied Organometallic Chemistry*, 5, 125–9.

Sanderson, J.T., Boerma, J., Lansbergen, G.W.A., and van den Berg, M. (2002) Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells, *Toxicology and Applied Pharmacology*, 182, 44–54.

Sasaki, K., Ishizaka, T., Suzuki, T., and Saito, Y. (1988) Determination of tri-*n*-butyltin and di-*n*-butyltin compounds in fish by gas chromatography with flame photometric detection, *Journal of the Association of Official Analytical Chemists*, 71, 360–3.

Scadding, S.R. (1990) Effects of tributyltin oxide on the skeletal structures of developing and regenerating limbs of the axolotl larvae, *Ambystoma mexicanum*, *Bulletin of Environmental Contamination and Toxicology*, 45, 574–81.

Schardein, J. (2000) Hormones and hormonal antagonists, in *Chemically Induced Birth Defects*, 3rd ed., revised and expanded, Marcel Dekker, New York.

Short, J.W. and Thrower, F.P. (1986) Accumulation of butyltins in muscle tissue of chinook salmon reared in sea pens treated with tri-*n*-butyltin, *Marine Pollution Bulletin*, 17, 542–5.

Smith, B.S. (1981a). Male characteristics on female mud snails caused by antifouling paints, *Journal of Applied Toxicology*, 1, 22–5.

Smith, B.S. (1981b). Tributyltin compounds induce male characteristics on female mud snails *Nassarius oboletus = Ilyanasa oboleta*, *Journal of Applied Toxicology*, 1, 141–4.

Snoeij, N.J., Penninks, A.H., and Seinen, W. (1987) Biological activity of organotin compounds—an overview, *Environmental Research*, 44, 335–53.

Snow, R.L. and Hays, R.L. (1983) Phasic distribution of seminiferous tubules in rats treated with triphenyltin compounds, *Bulletin of Environmental Contamination and Toxicology*, 31, 658–65.

Spencer, F. and Sing, L.T. (1982) Reproductive responses to rotenone during decidualized pseudogestation and gestation in rats, *Bulletin of Environmental Contamination and Toxicology*, 28, 360–8.

Spooner, N., Gibbs, P.E., Bryan, G.W., and Goad, L.J. (1991) The effect of tributyltin upon steroid titers in the female dogwhelk, *Nucella lapillus*, and the development of imposex, *Marine Environmental Research*, 32, 37–49.

Strmac, M. and Braunbeck, T. (1999) Effects of triphenyltin acetate on survival, hatching success, and liver ultrastructure of early life stages of zebrafish (*Danio rerio*), *Ecotoxicology and Environmental Safety*, 44, 25–39.

Stroben, E., Oehlmann, J., and Bettin, C. (1991) TBT-induced imposex and role of steroids in marine snails, in *Proceedings Tenth World Meeting of the Organotin Environmental Programme Association*, Berlin, September, 68–73.

Suzuki, T., Matsuda, R., and Saito, Y. (1992) Molecular species of tri-*n*-butyltin compounds in marine products, *Journal of Agricultural and Food Chemistry*, 40, 1437–43.

Takahashi, T., Araki, A., Nomura, Y., Koga M., and Arizono, K. (2000) The occurrence of dual-gender imposex in Japanese freshwater crab, *Journal of Health Science*, 46, 376–9.

Toyoda, M., Sakai, H., Kobayashi, Y., Komatsu, M., Hoshino Y., Horie, M., Saeki, M., Hasegawa, Y., Tsuji, M., Kojima, M., Toyomura, K., Kumano, M., and Tanimura, A. (2000) Daily dietary intake of tributyltin, dibutyltin, triphenyltin and diphenyltin compounds according to a total diet study in Japanese population, *Shokuhin Eiseigaku Zasshi*, 41, 280–6 (in Japanese).

Tryphonas, H., Cooke, G.M., Caldwell, D., Bondy, G., Parenteau, M., Hayward, S., and Pulido, O. (2004) Oral (gavage), in utero and postnatal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part II: Effects on the immune system, *Food and Chemical Toxicology*, 42, 221–235.

Tsuda, T., Inoue, T., Kojima, M., and Aoki, S. (1995) Daily intakes of tributyltin and triphenyltin comounds from meals, *Journal of AOAC International*, 78, 941–3.

Tsuda, T., Nakanishi, H., Aoki, S., and Takebayashi, J. (1987) Bioconcentration and metabolism of phenyltin chlorides in carp, *Water Research*, 21, 949–53.

Ueno, S., Susa, N., Furukawa, Y., Komatsu, Y., Koyama, S., and Suzuki, T. (1999) Butyltin and phenyltin compounds in some marine fishery products on the Japanese market, *Archives of Environmental Health*, 54, 20–5.

Waldock, M.J. and Thain, J.E. (1983) Shell thickening in Crassostrea gigas: organotin antifouling or sediment induced? *Marine Pollution Bulletin*, 14, 411–5.

Weis, J.S. and Kim, K. (1988) Tributyltin is a teratogen in producing deformities in limbs of the fiddler crab, *Uca pugilator*, *Archives of Environmental Contamination and Toxicology*, 17, 583–7.

Winek, C.L., Marks, M.J., Jr., Shanor, S.P., and Davis, E.R. (1978) Acute and subacute toxicology and safety evaluation of triphenyl tin hydroxide (Vancide KS), *Clinical Toxicology*, 13, 281–96.

Winship, K.A. (1988) Toxicity of tin and its compounds, *Adverse Drug Reactions and Acute Poisoning Reviews*, 7, 19–38.

World Health Organization (1980) Tin and Organotin Compounds: A Preliminary Review. *Environmental Health Criteria 15*, World Health Organization, Geneva.

World Health Organization (1992) Fentin, in *Pesticide Residues in Food 1991: Evaluations Part II Toxicology*, World Health Organization, Geneva. Online: http://www.inchem.org/documents/jmpr/jmpmono/v91pr11.htm (accessed 16 June 2004).

Yamabe, Y., Hoshino, A., Imura, N., Suzuki, T., and Himeno, S. (2000) Enhancement of androgen-dependent transcription and cell proliferation by tributyltin and triphenyltin in human prostate cancer cells, *Toxicology and Applied Pharmacology*, 169, 177–84.

Yonemoto, J., Shiraishi, H., and Soma, Y. (1993) In vitro assessment of teratogenic potential of organotin compounds using rat embryo limb bud cell cultures, *Toxicology Letters*, 66, 183–91.

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Mutation Research 588 (2005) 129–135

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In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals

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Received 12 June 2005; received in revised form 20 September 2005; accepted 26 September 2005
Available online 28 October 2005

Abstract

Genotoxicity is one of the important endpoints for risk assessment of environmental chemicals. Many short-term assays to evaluate genotoxicity have been developed and some of them are being used routinely. Although these assays can generally be completed within a short period, their throughput is not sufficient to assess the huge number of chemicals, which exist in our living environment without information on their safety. We have evaluated three commercially available in silico systems, i.e., DEREK, MultiCASE, and ADMEWorks, to assess chemical genotoxicity. We applied these systems to the 703 chemicals that had been evaluated by the Salmonella/microsome assay from CGX database published by Kirkland et al. [1]. We also applied these systems to the 206 existing chemicals in Japan that were recently evaluated using the Salmonella/microsome assay under GLP compliance (ECJ database). Sensitivity (the proportion of the positive in Salmonella/microsome assay correctly identified by the in silico system), specificity (the proportion of the negative in Salmonella/microsome assay correctly identified) and concordance (the proportion of correct identifications of the positive and the negative in Salmonella/microsome assay) were increased when we combined the three in silico systems to make a final decision in mutagenicity, and accordingly we concluded that in silico evaluation could be optimized by combining the evaluations from different systems. We also investigated whether there was any correlation between the Salmonella/microsome assay result and the molecular weight of the chemicals: high molecular weight (>3000) chemicals tended to give negative results. We propose a decision tree to assess chemical genotoxicity using a combination of the three in silico systems after pre-selection according to their molecular weight.
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Keywords: In silico; (Quantitative) structure-activity relationship; (Q)SAR; Chemical genotoxicity; Decision tree

1. Introduction

It is said that more than 20,000 chemicals are in use in Japan. Among them, only approximately 10% are thought to have been assessed for human hazard based on data from in vitro and in vivo bioassays. According to the "Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc." [2], the Salmonella/microsome (Ames) assay, in vitro chromosomal aberration assay (or alternatively mouse lymphoma TK assay), and 28-day repeat dose toxicity test in rodents are obligatory to notify new chemicals for production/import at a level of more than 10 t per year.

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doi:10.1016/j.mrgentox.2005.09.009

To screen the remaining 18,000 chemicals for human hazard by application of this three-test battery is not realistic from the time and economical point of view. We need a much higher-throughput system to assess these chemicals, at least for prioritization of those chemicals that should be submitted to biological testing. To assess human hazard for regulatory purposes, in silico systems are now beginning to be used [3]. Here, we evaluated three commercially available in silico (quantitative) structure-activity relationship ((Q)SAR) systems and tried to construct a decision tree for prioritization of which chemicals need in vitro and/or in vivo testing. Also, within the drug discovery process, integrated computational analysis has been proposed to be incorporated as a toxicity prediction tool [4].

Kirkland et al. [1] published a database (CGX database, see http://www.lhasalimited.org/cgx) for nearly 1000 carcinogens and non-carcinogens with results of representative in vitro genotoxicity assays, i.e., Salmonella/microsome assay (Ames), mouse lymphoma TK assay using L5178Y cells (MLA), and in vitro chromosomal aberration assay or in vitro micronucleus assay (CA/MN). We used 703 chemicals that had been assessed in the Ames assay for evaluation of the three in silico systems, i.e., DEREK, MultiCASE (MCase), and ADMEWorks (AWorks). We also used a database (the ECJ database) that we constructed from chemicals existing in Japan that had recently been assessed in the Ames assay, in vitro chromosomal aberration assay, and 28 day repeat dose rodent toxicity test and/or reproductive and developmental toxicity test for their safety evaluation under GLP compliance. The ECJ database consisted of 206 chemicals but only 26 chemicals were positive by the Ames assay. Initially we evaluated both sensitivity and specificity of these three systems using the ECJ database of 206 chemicals [5].

We selected these three in silico systems because of their different modes of analysis. DEREK is a rule-based system [6], MCase [7] is a database/substructure based system, and AWorks is a QSAR. We applied these systems individually to assess gene-mutation induction on the 703 and 206 chemical sets described above and evaluated their sensitivity, specificity, concordance, and applicability (how many chemicals could be assessed), independently.

It is known that high molecular weight polymers tend not to induce gene mutation and chromosomal aberrations mainly because they cannot enter the target cells to react with DNA, or other bio-molecules necessary for genetic stability. We analyzed 194 Ames positive chemicals (confidential source) for the effect of molecular weight.

2. Materials and methods

2.1. Data sources for chemicals assessed

Of about 1000 chemicals, 703 that had been assessed in the Ames test were chosen from the CGX database published by Kirkland et al. [1]. All chemical structures were re-drawn using Chemdraw Ultra (Cambridge Soft Corporation, USA) and converted to MOL files before application to each system. We also used the database of 206 chemicals evaluated in the MHLW project "Safety Examination of Existing Chemicals and Safety Programmes in Japan" (ECJ database). The test summary for each of these chemicals can be seen at http://wwwdb.mhlw.go.jp/ginc/html/db1.html. In addition, we collected 194 Ames positive chemicals from a confidential source and investigated the relationship between gene mutation induction and molecular weight, with identification of any active side chain that might have contributed to the positive result in the Ames assay.

2.2. In silico systems used and definition of positive and negative responses

We used DEREK (Lhasa Ltd., UK) version 8.0.1.When the system gave an evaluation as "certain", "probable" or "plausible" we considered this as "positive", and when the system gave "equivocal", "doubted", "improbable", "impossible", or "no alert" we considered this as "negative". We used MCase (Multicase Co. Ltd.) version mc4pc. When the system gave "active" or "marginal" we considered this as "positive", and when the system gave "Inactive" we considered this as "negative". In the case of AWorks (Fujitsu Kitakyushu, Co. Ltd., version 2.0), we considered as "positive" when system evaluation was "positive", and considered as "negative" when the system evaluation was "negative". We excluded chemicals from further analysis when DEREK or AWorks gave no answer, or the evaluation was "inconclusive" by MCase.

2.3. Definition of sensitivity, specificity, concordance, and applicability

We calculated sensitivity, specificity, concordance, and applicability as follows:

$$\text{sensitivity} = \frac{N_{\text{A+S+}}}{N_{\text{A+}}} \times 100, \qquad \text{specificity} = \frac{N_{\text{A-S-}}}{N_{\text{A-}}} \times 100,$$

$$\text{concordance} = \frac{N_{\text{A+S+}} + N_{\text{A-S-}}}{N_{\text{eval}}} \times 100,$$

$$\text{applicability} = \frac{N_{\text{eval}}}{N_{\text{all}}} \times 100$$

where $N_{\text{A+}}$ is number of chemicals revealing positive in Ames assay; $N_{\text{A-}}$ is number of chemicals negative in Ames assay; $N_{\text{A+S+}}$ is number of chemicals revealing positive by both Ames assay and in silico evaluation; $N_{\text{A-S-}}$ is number of chemicals negative in both Ames assay and in silico evaluation; N_{eval} is

Table 1
Performance of in silico systems

	Ames result	+	−	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
CGX database								
DEREK	+	288	64	352				
	−	69	267	336	81.8	79.5	80.7	97.9
	Total	357	331	688				
MCase	+	235	32	267				
	−	6	249	255	88.0	97.6	92.7	74.3
	Total	241	281	522				
AWorks	+	267	89	356				
	−	149	187	336	75.0	55.7	65.6	98.4
	Total	416	276	692				
ECJ database								
DEREK	+	19	7	26				
	−	21	159	180	73.1	88.3	86.4	100.0
	Total	40	166	206				
MCase	+	13	7	20				
	−	13	133	146	65.0	91.1	88.0	80.6
	Total	26	140	166				
AWorks	+	19	7	26				
	−	54	124	178	73.1	69.7	70.1	99.0
	Total	73	131	204				

MCase: MultiCASE; AWorks: ADMEWorks.

number of chemicals evaluated; and N_{all} is total number of chemicals subjected.

3. Results

Among the set of 703 CGX chemicals with published Ames data, 358 were positive and 345 were negative. The results of the in silico evaluation are summarized in Table 1. The highest sensitivity, specificity, and concordance with Ames assay results was provided by MCase, then followed by DEREK. However, the systems that showed the best applicability were AWorks and (almost the same) DEREK, then followed by MCase. For the database of 206 ECJ chemicals, 26 were positive and 180 were negative. The outcomes of the in silico analyses are summarized in Table 1. The pattern of performance was very similar to that with the 703 chemicals in the CGX database.

Fig. 1 shows the cumulative percent of Ames positive chemicals against molecular weight. It can be seen that 87.1% of those positive chemicals had molecular weights less than 1000, and 96.4% had molecular weights less than 3000; in other words, only 3.6% of the chemicals with a molecular weight >3000 gave a positive response in the Ames assay. Seven of 194 Ames positive chemicals had a molecular weight >3000 and four of these seven polymers had epoxy groups.

When we combined the in silico systems, the performance was different from that when assessed individually (Table 2). If we considered the in silico mutagenicity as positive (or negative) when two or more systems gave positive (or negative) evaluations, 87.8

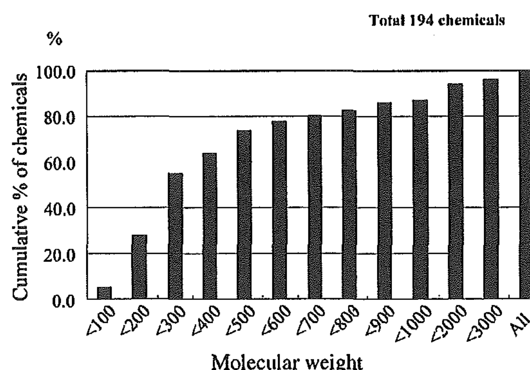

Fig. 1. Cumulative percentage of chemicals based on their molecular weight. 194 Ames positive chemicals were analyzed. 7/194 chemicals were more than 3000 molecular weight and Ames positive and 4/7 contained epoxy groups.

Table 2
Performance of in silico systems after combined

CGX database

Ames \ In silico	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	279	40	319				
-	42	249	291	**87.8**	**85.6**	**86.7**	**86.8**
Total	321	289	610				
	+++	---					
+	166	1	167				
-	3	127	130	**99.4**	**97.7**	**98.7**	**42.2**
Total	168	129	297				

ECJ database

Ames \ In silico	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	19	7	26				
-	23	147	170	**73.1**	**86.5**	**84.7**	**95.1**
Total	42	154	196				
	+++	---					
+	13	2	15				
-	5	94	99	**86.7**	**94.9**	**93.9**	**55.3**
Total	18	96	114				

Table 3
Performances of DEREK and MCase in several published papers.

Target compounds	In silico system	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	Reference
394 Drugs	DEREK	52	75	74	94[a]	[11]
	MCase	48	93	90	91[a]	
217 Non-drugs	DEREK	86	50	81	100[a]	[10]
	MCase	91	62	83	100[a]	
520 Drug candidates	DEREK	28	80	72	100	[13]
	MCase	50	86	81	41	
	DEREK + MCase	29	95	88	29	
	DEREK + Mcase + TOPKAT	75	96	95	15	
123 Drug candidates	DEREK	8[b]	31[c]	61	100[d]	[4]
	MCase (A2H)	13[b]	15[c]	72	97[d]	
	Topcat (Ames Mut)	18[b]	15[c]	67	98[d]	
	DEREK + MCase	6[b]	19[c]	75	97[d]	
	DEREK + Mcase + TOPKAT	5[b]	9[c]	86	46[d]	
94 Non-drugs	DEREK	63	81	76	100	[13]
	MCase	40	90	76	75	
	DEREK + MCase	47	100	85	56	
	DEREK + Mcase + TOPKAT	55	100	86	37	
516 Non-drugs	DEREK	6[b]	24[c]	70	100[d]	[4]
	MCase (A2H)	7[b]	12[c]	81	98[d]	
	Topcat (Ames Mut)	25[b]	19[c]	56	97[d]	
	DEREK + MCase	2[b]	16[c]	82	98[d]	
	DEREK + Mcase + TOPKAT	7[b]	10[c]	83	43[d]	

[a] Calculated by us
[b] % False negative.
[c] % False positive.
[d] (1-Indeterminate).

and 73.1% sensitivity, 85.6 and 86.5% specificity, 86.7 and 84.7% concordance, and 86.8 and 95.1% applicability were obtained for the CGX and ECJ databases, respectively. If we considered the in silico mutagenicity as positive (or negative) only when all three systems gave positive (or negative) evaluations, all performance measures (sensitivity, specificity, etc.) increased up to 98.7 and 93.9%. However, applicability decreased to 42.2 and 55.3%, which meant only about half of the chemicals in the CGX and ECJ databases could be evaluated. One chemical, *o*-phenylphenol [90-43-7], was positive in the Ames test but negative by all three in silico systems and three chemicals, carboxymethylnitrosourea [60391-92-6], methidathion [950-37-8], 1-nitroso-3,5-dimethyl-4-benzoylpiperazine [61034-40-0], were negative in the Ames test although all three in silico system gave positive evaluation for mutagenicity in the CGX database. When we used the ECJ database, 2-amino-1-naphthalenesulfonic acid [81-16-3] and 2-vinylpyridine [100-69-6] were positive in the Ames test but negative by all three in silico systems and there was no chemical that was negative in the Ames assay and all positive in in silico system. These exceptional chemicals are listed in Table 3 together with such chemicals taken from literatures.

4. Discussion

It is important to construct a strategy for efficient evaluation of the toxicity of a large number of existing chemicals. Even so-called short-term assays, e.g., Ames assay and in vitro chromosomal aberration assay, can practically assess only 100 chemicals per year according to our experiences in Japan. In this case, it will take 180 years to assess the outstanding 18,000 existing chemicals for genotoxicity, and it will take even longer when repeat dose toxicity tests are also performed, as these are not short-term assays. We therefore need higher-throughput systems to assess chemical safety, or at least to set priorities for those chemicals that should be tested in in vitro and/or in vivo tests. In silico systems have the capability for high throughput but have not yet been well validated for assessment of human hazard, although some regulatory bodies have started to use these methods.

Correlation between the Ames assay result and molecular weight could be explained by the lack of membrane permeability of high molecular weight chemicals, making it more difficult for them to reach target molecules such as DNA and proteins that contribute to the fidelity of cell division. Therefore, only a few chemicals with molecular weight >3000 gave positive responses in the Ames assay. This phenomenon is also true for induction of chromosomal aberrations in vitro (data not shown). The other important issue is the contribution of epoxy group in the polymer. Although of molecular weight >3000, some polymers with an epoxy group gave positive results in both the Ames and chromosomal aberration assays. Epoxy embedding reagents employed in electron microscopy (e.g., epon and araldite) have been reported as mutagenic in the Ames assay [8]. According to these findings, we should include a step to evaluate molecular weight and existence of any epoxy groups in the molecule.

In the present study, we used the CGX database recently published by Kirkland et al. [1] for microbial mutagenicity data on 358 carcinogens and 345 non-carcinogens for validation of three commercially available in silico (Q)SAR systems. When applied individually, MCase gave high sensitivity, specificity, and concordance compared to other two systems. One of the reasons may be because the CGX database contained many results from the U.S. National Toxicology Program (NTP), and the learning dataset of MCase would have used many of the same results. Therefore, some of them were evaluated by direct matching. Moreover, the applicability of MCase was relatively low compared with the other systems in this study (Table 1). MCase judged 119 chemicals as inconclusive and one chemical as marginal, and could not evaluate 67 chemicals. Such selectivity in MCase may contribute to the high concordance. On the other hand, the other systems were not influenced directly by the NTP data. We applied the in silico systems to another dataset, the ECJ database, that does not contain the NTP data and we obtained similar patterns of sensitivity, specificity, etc.

Each in silico system showed different outcomes on some chemicals complimentary by some extent. These different evaluation patterns were mainly due to the different evaluation rules. The DEREK is a rule-based system, AWorks is a discriminant-based system mainly depending on physicochemical descriptors, and MCase is a hybrid system based on a database. Therefore, we concluded that in silico evaluation could be optimized by combining the evaluations from the three systems. Sensitivity, specificity and concordance were increased when we combined the three in silico systems to make a final conclusion of mutagenicity (Table 1). Concordance was much higher after combining but the applicability became poor (42.2%). When two of the in silico systems gave the same evaluations, the applicability (86.8%) was good but the concordance was lower (86.7%) than when all three were combined (98.7).

Recently, several in silico studies for prediction of mutagenicity have been conducted on drugs or non-

Table 4
Exceptional chemicals that showed Ames test gave positive but all three in silico systems (DEREK, MCase, TOPKAT/AWorks) gave negative and Ames test gave negative but all three systems gave positive

Compound	CAS	Ames test	DEREK	MCase	TOPKAT/Aworks	Source[a]
Bupropion	34911-55-2	+	–	–	–	1
Citalopram	59729-33-8	+	–	–	–	1
Naloxone	465-65-6	+	–	–	–	1
Oxcarbazepime	28721-07-5	+	–	–	–	1
Quetiapine	111976-69-7	+	–	–	–	1
Rabeprazole	117976-89-3	+	–	–	–	1
Zolmitriptan	139264-17-8	+	–	–	–	1
2-(2-Methylpropyl) thiazole	18640-74-9	+	–	–	–	2
2-Chloropyridine	109-09-1	+	–	–	–	2
Pyrogallol	87-66-1	+	–	–	–	2
o-Phenylphenol	90-43-7	+	–	–	–	3
2-Amino-1-naphthalenesulfonic acid	81-16-3	+	–	–	–	3
2-Vinylpyridine	100-69-6	+	–	–	–	3
Fosfomycin	23155-02-4	–	+	+	+	1
Toremifene	89778-26-7	–	+	+	+	1
Poly (2-hydroxypropyl methacrylate)	25703-79-1	–	+	+	+	2
Carboxymethylnitrosourea	60391-92-6	–	+	+	+	3
Methidathion	950-37-8	–	+	+	+	3
1-Nitroso-3,5-dimethyl-4-benzoylpiperazine	–	+	+	+	3	

[a] 1: Synder et al. [11] (with TOPKAT), 2: White et al. [13] (with TOPKAT), 3: this study (with AWorks).

drug chemicals with commercially available programs, e.g., DEREK, MCase or TOPKAT, or newly developed computational approaches [4,9–12]. The performances of DEREK and MCase in several of these studies are summarized in Table 4. Generally, similar performance in sensitivity, specificity, concordance, and applicability were shown between DEREK and MCase but with some exceptions, e.g., sensitivity in 520 drug candidates [13], specificity in 516 non-drugs [4], and applicability in 520 pharmaceutical drug candidates and 94 non-drugs [13]. These differences might be due to the chemical class of target compounds in each database. However, there was no remarkable difference in performance whether the chemical was intended for use as a pharmaceutical, agricultural, or industrial agent. Our results on performance of in silico systems showed similarity with the published analyses. With respect to the combination of in silico prediction systems, White et al. [13] reported that combination improved the overall accuracy and specificity, but sensitivity was barely above the 50% level (Table 4). On the other hand, their analysis showed quite low applicability in the combination of three prediction systems, DEREK, MCase and TOPKAT. Our analysis of the combination of DEREK, MCase and AWorks showed good improvements in sensitivity, specificity and concordance, but applicability was low, especially in the 3-system combination.

Exceptional chemicals that gave positive Ames results but were negative in all three in silico systems (DEREK, MCase, TOPKAT/AWorks), and those that were negative in the Ames test but gave positive evaluations in all three systems, are summarized in Table 4. This table, which includes data from Synder et al. [11] and White et al. [13] shows there are 19 exceptional chemicals from both drug and non-drug families. Although it would be unrealistic to expect zero exceptions using this approach, further improvement of the prediction systems is needed. We do not have good reasons to explain the discordance, therefore we will verify the results from both sides, i.e., in silico system and Ames test.

Considering these outcomes, we propose a decision tree (Fig. 2), in order to evaluate chemical induction of gene mutation. We may use the decision tree to prioritize chemicals to be assayed by in vitro and/or in vivo tests. A final goal being that eventually, chemical mutagenicity will be evaluated by in silico systems alone for regulatory use. The decision tree consists of three steps; namely to assess the molecular weight, the existence of epoxy groups, and the in silico evaluation for genotoxicity. Based on the purpose of the in silico evaluation, the tree might be altered by the different final call of the in silico evaluation, i.e., regarding as positive (negative) all three systems show positive (negative). The choice of definition for final call applying to the decision tree should be based on the balance between accuracy of eval-

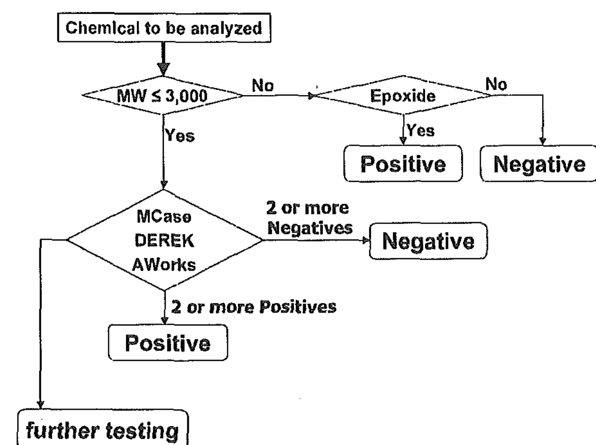

Fig. 2. Decision tree. In in silico evaluation, when two or more give positive then the final call is "positive" and two or more negative then call "negative".

uation and applicability, which are especially important for regulatory purpose. The decision should be made on a case-by-case basis depending upon the purpose of the decisions to be made.

Acknowledgements

The authors thank Dr. D. Kirkland, Covance, for his critical review and kind English edition and Mr. T. Ehara, MHLW, for his invaluable discussion. Authors also want to thank Ms. H. Akiyama (CTC laboratories, Japan), Akamatsu and Naitoh (Charles River Japan, Japan), and Kitajima, Suiroi, and Yuta (Fujitsu Kitakyushu, Japan) for their technical assistance. This work supported by the Health and Labour Sciences Research Grants (H15-Chemistry-003).

References

[1] D. Kirkland, M. Aardema, L. Henderson, L. Müller, Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity, Mutat. Res. 584 (2005) 1–256.

[2] Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc., Law No. 117, 16 October 1973 as last amended by Law No.49, 28 May 2003.

[3] M.T.D. Cronin, J.S. Jaworska, J.D. Walker, M.H.I. Comber, C.D. Watts, A.P. Worth, Use of QSARs in international decision-making frameworks to predict health effects of chemical substances, Environ. Health Perspect. 111 (2003) 1391–1401.

[4] G.M. Pearl, S. Livingstone-Carr, S.K. Durham, Integration of computational analysis as sentinel tool in toxicological assessments, Curr. Topoics Med. Chem. 1 (2001) 247–255.

[5] A. Hirose, M. Takahashi, M. Kamata, M. Ema, M. Hayashi, Development of genotoxicity predicting QSAR system for registered and existing industrial chemicals in Japan, Toxicol. Appl. Pharmacol. 197 (2004) 358.

[6] N. Greene, P.N. Judson, J.J. Langowski, C.A. Marchant, Knowledge-based expert systems for toxicity and metabolism prediction: DEREK, StAR and METEOR, SAR QSAR Environ. Res. 10 (1999) 299–314.

[7] H.S. Rosenkranz, A.R. Cunningham, Y.P. Zhang, H.G. Claycamp, O.T. Macina, N.B. Sussman, S.G. Grant, G. Klopman, Development, characterization and application of predictive-toxicology models, SAR QSAR Environ. Res. 10 (1999) 277–298.

[8] M.P. Murray, J.E. Cummins, Mutagenic activity of epoxy embedding reagents employed in electron microscopy, Environ. Mutagen. 1 (1979) 307–313.

[9] N.F. Cariello, J.D. Wilson, B.H. Britt, D.J. Wedd, B. Burlinson, V. Gombar, Comparison of the computer programs DEREK and TOPKAT to predict bacterial mutagenicity. Deductive estimate of risk from existing knowledge. Toxicity prediction by computer assisted technology, Mutagenesis 17 (4) (2002) 321–329.

[10] J.R. Votano, M. Parham, L.H. Hall, L.B. Kier, S. Oloff, A. Tropsha, Q. Xie, W. Tong, Three new consensus QSAR models for the prediction of Ames genotoxicity, Mutagenesis 19 (5) (2004) 365–377.

[11] R.D. Snyder, D.E. Ewing, L.B. Hendry, Evaluation of DNA intercalation potential of pharmaceuticals and other chemicals by cell-based and three-dimensional computational approaches, Environ. Mol. Mutagen. 44 (2) (2004) 163–173.

[12] R.D. Snyder, G.S. Pearl, G. Mandakas, W.N. Choy, F. Goodsaid, I.Y. Rosenblum, Assessment of the sensitivity of the computational programs DEREK, TOPKAT, and MCASE in the prediction of the genotoxicity of pharmaceutical molecules, Environ. Mol. Mutagen. 43 (3) (2004) 143–158.

[13] A.C. White, R.A. Mueller, R.H. Gallavan, S. Aaron, A.G. Wilson, A multiple in silico program approach for the prediction of mutagenicity from chemical structure, Mutat. Res. 539 (1–2) (2003) 77–89.

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Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)—Mammalian Mutagenicity Study Group (MMS)

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Received 25 August 2004; received in revised form 18 February 2005; accepted 15 March 2005

Abstract

We conducted simultaneous liver and peripheral blood micronucleus assays in young rats with seven rodent hepatocarcinogens—4,4′-methylenedianiline (MDA), quinoline, *o*-toluidine, 4-chloro-*o*-phenylenediamine (CPDA), dimethylnitrosamine (DMN), *p*-dimethylaminoazobenzene (DAB), and di(2-ethylhexyl)phthalate (DEHP)—and two mutagenic chemicals—kojic acid and methylmethanesulfonate (MMS).

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doi:10.1016/j.mrgentox.2005.03.012

Quinoline, DMN, and DAB were positive in the liver assay, while *o*-toluidine, kojic acid, DAB, and MMS were positive in the peripheral blood assay. *o*-Toluidine, kojic acid, and DAB are reportedly negative in mouse bone marrow micronucleus assays, indicating a species difference.
Our results revealed a correlation between micronucleus induction in hepatocytes and hepatocarcinogenicity. This technique can be useful for the detection of micronucleus-inducing chemicals that require metabolic activation, and it enables simultaneous comparison of the micronucleus-inducing potential of chemicals in the liver and peripheral blood in the same individual.
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Keywords: Young rat; Liver micronucleus; Peripheral blood micronucleus; Hepatocarcinogen

1. Introduction

In vivo rodent bone marrow (BM) micronucleus assay results correlate highly with carcinogenicity in many organs, but the test is rather insensitive to indirect and liver carcinogens [1]. The micronucleus-inducing potential of such chemicals can be detected in in vivo liver micronucleus assays [2–4], which can be conducted by the partial hepatectomy (PH) method [2,5,6], co-treatment with mitogens [7,8] or an in vivo/in vitro assay system [9]. These all have serious disadvantages. In the PH method, P-450, styrenemonooxygenase, epoxide hydrolase, and glutathione-*S*-epoxide transferase activity is decreased [10], and the method is time-consuming because it involves surgery. In the co-treatment method with mitogens, the mitogens can interact with the test chemicals [11]. The in vivo/in vitro assay system requires much effort, time, and expense.

Searching for better approach, we evaluated liver micronucleus assay that uses 4-week-old rats [11]. We evaluated the assay using the hepatocarcinogen diethylnitrosamine (DEN) [12]. In 4-week-old rats, not only liver growth but also P450 activity are at their maximum and glucuronic acid, sulfate, glutathione, and glycine conjugation levels are the same as in mature animals [13], as are the levels of hexobarbital hydroxylation, *N*-demethylation of ethylmorphine, *O*-demethylation of *p*-nitroanisole and hydroxylation of aniline [14]. Since the usefulness of this method has not been clearly demonstrated, we organized a collaborative study to evaluate it with nine model chemicals. We conducted the peripheral blood micronucleus assay [15,16] simultaneously to evaluate another organ in the same animal. Our results demonstrated the relationship in young rats between the hepatocarcinogenicity and hepatocyte micronucleus-inducing potential of the test chemicals.

2. Materials and methods

2.1. Collaboration

Eleven research laboratories collaborated in this study (Table 1).

2.2. Animals

Male Fischer F344 or SD rats, 3 weeks of age, were purchased from Charles River Japan Inc., and used at 4 weeks of age. The animals were housed under a 12-h light–dark cycle and allowed free access to commercial pellets and tap water.

2.3. Chemicals

4,4′-Methylenedianiline (MDA, CAS No. 101-77-9), kojic acid (CAS No. 501-30-4), quinoline (CAS No. 91-22-5), *o*-toluidine (CAS No. 95-53-4), 4-chloro-*o*-phenylenediamine (CPDA, CAS No. 95-83-0), and dimethylnitrosamine (DMN, CAS No. 62-75-9) were purchased from Wako Pure Chemical Industries Ltd.; *p*-dimethylaminoazobenzene (DAB, CAS No. 60-11-7), di(2-ethylhexyl)phthalate (DEHP, CAS No. 117-81-7), and methylmethanesulfonate (MMS, CAS No. 66-27-3) from Aldrich. Diethylnitrosamine (DEN, CAS No. 55-18-5) was purchased from Wako Pure Chemical Industries Ltd. or Tokyo Kasei Co. Ltd., and cyclophosphamide (CP, CAS No. 50-18-0) was purchased from ICN Biochemicals or Aldrich.

MDA, *o*-toluidine, CPDA, and DAB were suspended in olive oil, quinoline and DEHP in corn oil. Kojic acid was suspended in 1% sodium carboxymethylcellulose. DMN was dissolved in distilled water, MMS in physiological saline. DEN and CP, the positive control substances, were dissolved in distilled water, and the same lot chemical was used in all laboratories.

Table 1
Study participants

	Laboratory	Investigators
1	Biosafety Research Center, Foods, Drugs and Pesticides	Jin Tanaka
2	Hokko Chemical Industry Co. Ltd.	Yasushi Shimada
3	Ina Research Inc.	Hiroshi Suzuki[a], Kana Komatsu Akiko Koeda, Tadashi Imamura
4	Kaken Pharmaceutical Co. Ltd.	Junichi Yoshida
5	Kao Corporation	Naohiro Ikeda
6	Kissei Pharmaceutical Co. Ltd.	Kazuo Kobayashi, Yukari Terashima, Kaori Yasue
7	Mitsubishi Chemical Safety Institute Ltd.	Yukiko Saito
8	National Institute of Health Sciences	Takayoshi Suzuki, Makoto Hayashi
9	Nisshin Kyorin Pharmaceutical Co. Ltd.	Shigeki Hatakeyama
10	Sankyo Co. Ltd.	Toshiyuki Hagiwara, Ayumi Okazaki
11	Toa Eiyo Ltd.	Koko Nagaoka

[a] Chief study organizer.

2.4. Doses

We used 1/2 and 1/4 of the LD_{50} value of each chemical as the high and low dose. When the LD_{50} values were unclear, we estimated them by small-scale experiments according to the method of Lorke [17]. Negative control animals received the respective vehicle. Positive control animals received DEN at 40 mg/kg (liver micronucleus assay) or CP at 10 mg/kg (peripheral blood micronucleus assay). Each group consisted of four or five animals. Dosing was conducted once intraperitoneally or orally. With the exception of MMS, each chemical was evaluated by two laboratories.

2.5. Liver micronucleus assay

Rats were anesthetized with ethylether 3, 4 or 5 days after a single administration of test chemical or 5 days after administration of the negative or positive control chemicals. Hepatocytes were isolated by the collagenase perfusion method, rinsed with 10% neutral formalin two or three times, centrifuged at 50 × g for 1 min, suspended in 10% neutral formalin, and stored under refrigeration. For staining, 10–20 μL of the suspension was mixed with an equal volume of acridine orange (AO)–4′6-diamidino-2-phenylindole dihydrochloride (DAPI) [12]. Approximately 10–20 μL of stained suspension was dropped onto a clean glass slide and covered with a cover slip (24 mm × 40 mm).

Microscopic preparations were evaluated with the aid of a fluorescence microscope (×400 or greater) with UV excitation. The number of micronucleated hepatocytes (MNHEPs) among 2000 hepatocytes (two fields) was recorded for each animal. MNHEPs were defined as hepatocytes with round or distinct micronuclei that stained like the nucleus, with the ≤1/4 diameter of the nucleus [7,18]. The number of mitotic cells per 2000 hepatocytes was determined.

2.6. Peripheral blood micronucleus assay

A small amount of blood was collected from a tail vessel on Day 2 after treatment, at which time most chemicals induce the maximum response [19]. It was stained by either of the following methods: (1) 5–10 μL was dropped on to AO-coated slides, covered with cover glasses, and stored in a deep freezer until analysis [15], or (2) 10 μL suspension was mixed with about 30 μL of 10% neutral formalin and stored at room temperature, the samples were mixed with an equal volume of AO solution (500 μg/mL) in the ratio of 1:1 and smeared on a glass slide immediately before analysis. Specimens were evaluated with the aid of a fluorescent microscope (×600 or greater) with B excitation. The number of micronucleated reticulocytes (MNRETs) among 2000 reticulocytes (RETs) and the number RETs among 1000 erythrocytes were recorded for each animal.

2.7. Statistical analysis

We determined the statistical significance of the incidence of micronucleated hepatocytes or reticulocytes using Kastenbaum and Bowman's method [20] and that of reticulocytes with the Student t-test.

3. Results

3.1. Liver micronucleus assay

Table 2 shows the results of the liver micronucleus assay. Quinoline, DMN, and DAB were positive in both