Chemical	Maximum conc[a,j]	Guideline value[a]	Chemical	Maximum conc[a,j]	Guideline value[a,b]
(PCB156)		ng/L[i]			
2,3,3',4,4'-pentachlorobiphenyl (PCB105)	0.027 ng/L	0.016 ng/L[i]	Diatrizoic acid	1900 ng/L	350 ng/L[g]
2,3',4,4',5-Pentachlorobiphenyl (PCB118)	0.064 ng/L	0.016 ng/L[i]	Dibutyltin (DBT)	34 ng/L	2 ug/L[f]
2,4,5,3',4',5'-Hexachlorobiphenyl (PCB167)	0.004 ng/L	0.016 ng/L[i]	Di-n-butyl phthalate	891 ng/L	35 ug/L[f]
2,6-di-tert-butyl-1,4-benzoquinone (2,6-bis(1,1-dimethylethyl)-2,5-Cyclohexadiene-1,4-dione)	460 ng/L	140 ng/L[f]	4-Methylphenol (p-cresol)	0.54 ug/L	0.6[g]
2,6-di-tert-butylphenol (2,6-bis(1,1-dimethylethyl)phenol)	110 ng/L	2000 ng/L[f]	Monobutyltin (MBT)	90 ng/L	700 ng/L[g]
2,7-Dichlorodibenzo-p-dioxin (DCDD)	1200 ng/L	0.016 ng/L[i]	Naphthalene	80 ng/L	70 ug/L[f]
3,4,5,3',4',5'-Hexachlorobiphenyl (PCB169)	0.002 ng/L	0.016 ng/L[f]	N-nitrosomorpholine (NMOR)	12 ng/L	1 ng/L[f]
4-Chlorophenol	16 ng/L	10 ug/L[f]	Octachlorodibenzo-p-dioxin (OCDD)	0.1 ng/L	0.016 ng/L[i]
4-cumylphenol	0.98 ug/L	0.35 ug/L[f]	PCB77	0.006 ng/L	0.016 ng/L[i]
4-Nonylphenol (4NP)	185 ng/L	500 ug/L[f]	Phenanthrene	3000 ng/L	150 ug/L[f]
4-tert octylphenol	14 ng/L	50 ug/L[f]	Phenol	1300 ng/L	150 ug/L[f]
Anthracene	110 ng/L	150 ug/L[f]	Phthalic anhydride	1000 ng/L	7[f]
Acetophenone	410 ng/L	400 ug/L[f]	Pyrene	840 ng/L	150 ug/L[f]
Benzo(a)pyrene	240 ng/L	10 ng/L[h]	Stigmastanol	4 ug/L	10[h]
Benzyl chloride	1.8 ng/L	200 ng/L[f]	Tributyl phosphate	190 ng/L	500 ng/L[g]
Bisphenol A	32 ng/L	200ug/L[f]	Tributyltin (TBT)	21 ng/L	1000 ng/L[g]
Butylated hydroxytoluene (2,6-Di-tert-Butyl-p-Cresol)	100 ng/L	1[f]	Tri(butyl cellosolve) phosphate (ethanol,2-butoxy-phosphate)	6700 ng/L	50 ug/L[f]
Butylated hydroxyanisole (3-tert-butyl-4-hydroxy anisole)	200 ng/L	1.8[f]	Triclosan	0.4 ug/L	0.35 ug/L[g]
Caffeine	44 ug/L	0.35 ug/L[g]	Triphenyl Phosphate	220 ng/L	1000 ng/L[g]
Chlorophene	710 ng/L	350 ng/L[g]	Tris(2-chloroethyl)phosphate	540 ng/L	1000 ng/L[g]
Cholesterol	10 ug/L	7 ug/L[g]			
Radiological					
Alpha particles	0.7 Bq/L	0.5 Bq/L[b]	Beta particles and photon emitters	1.2 Bq/L	0.5 Bq/L[b]
Gross gamma	0.1 Bq/L	0.5 Bq/L			
Chelating agents					
Ethylenediaminetetraacetic acid (EDTA)	0.210	0.25[b]	PDTA (Propylenedinitrilo)tetraacetic acid)	0.027	0.0007[g]
Nitrilotriacetic acid (NTA)	0.012	0.2[b]			

aesth = aesthetic guideline - no health guideline value

a Values expressed as mg/L unless otherwise indicated
b NHMRC–NRMMC (2004)
c EC 98/83/EC
d US EPA (2007)
e *Guidelines for Drinking-water Quality* (WHO 2006) (corrected to apply carcinogenicity risk of 10^{-6})
f Published total daily intake or equivalent (Appendix A)
g Calculated value (Appendix A)
h Calculated from therapeutic doses or ADI (Appendix A)
i Compounds with dioxin-like activity should provide a total of <16 pg toxic equivalent per litre taking into account toxicity equivalence factors (NHMRC 2002) (Appendix A)
j Maximum concentrations were obtained from unpublished Australian data and from Costanzo and Watkinson (2007), Kolpin et al (2002), Fatta et al (2006), Castillo et al (1997), Gomez et al (2007), Fent (1996), Daughton and Ternes (1999).

Hazards to the environment

Indirect drinking water augmentation incorporates the discharge of treated recycled water into receiving waters such as rivers, reservoirs, streams and aquifers. The recycled water discharged to receiving waters will be subject to greater levels of treatment and will be of a much higher quality than recycled water discussed in Phase 1 of the water recycling guidelines (NRMMC–EPHC

Appendix A Setting guidelines for chemicals in drinking water augmented with recycled water

A1 Overview

Whatever the source of water — treated sewage, stormwater or traditional sources such as rivers, reservoirs or groundwater — it will contain a variety of chemicals. This appendix explains the process for setting guidelines to protect human health from chemicals in drinking water when recycled water is used as the source. The process described in this appendix was used to set the drinking water guidelines given in Table 4.4 (Chapter 4); Box A1 explains what is meant by the term 'drinking water guideline'.

The primary focus of the approach described here is augmentation of drinking water with treated sewage. However, the outcomes could be applied to drinking water produced from any raw water supply, such as stormwater, reservoirs, rivers, groundwater, rain water, industrial waste water and mine waters.

Unlike the Australian Drinking Water Guidelines (ADWG) (NHMRC–NRMMC 2004), aesthetic considerations of taste are not considered in the guidelines given here.

Box A1 Drinking water guideline

Throughout this appendix, the term 'drinking water guideline' refers to a concentration of chemical in drinking water delivered to the consumer that may, either in whole or in part, include recycled water. The Australian Drinking Water Guidelines (NHMRC–NRMMC 2004) explains the rationale behind a guideline value for a particular chemical as follows:

> the concentration that, based on present knowledge, does not result in any significant risk to the health of the consumer over a lifetime of consumption and is consistent with water of good quality. The health related guideline values are very conservative, and are calculated using a range of safety factors. They always err on the side of safety, particularly where scientific data are inconclusive or where the only data available are from animal studies.'

In other words, if the water complies with the drinking water guidelines, then drinking water containing recycled water is safe to drink. Short periods of consuming water containing chemicals at concentrations higher than the guideline values do not necessarily equate with a high likelihood of adverse health effects. The probability of an adverse health effect depends mainly on the actual concentration of chemical in the water and the length of time it was consumed.

The drinking water guidelines recommended here for chemicals have the 'end-of-pipe' consumer as the target receptor. The overriding philosophy applied in this document is that drinking water produced from source water that may contain recycled water should be at least as safe as that from traditional water sources. Consequently, the recommended guidelines have been established in a way that is consistent with the approach currently used in Australia and internationally for setting health-protective guidelines for chemicals potentially found in food, water or air. The main focus of this appendix is the process for setting guidelines for chemicals for which no drinking water guideline is available.

A2 Process for setting guidelines

Figure A1 summarises the process for setting drinking water guidelines as a hierarchical decision tree. This section of the appendix discusses each of the steps outlined in the diagram.

A2.1 Step 1 — List chemicals of interest

The first step in the decision tree for setting drinking water guidelines is to list chemicals of interest. These could include chemicals that have been found in the effluent of secondary sewage treatment either in Australia or overseas (it is assumed that sewage used as source of recycled water to augment drinking water supplies will be subject to secondary treatment) and chemicals of general interest to the community.

Table 4.4 in Chapter 4 provides data from secondary treated effluent from a range of Australian treatment plants and published international reports. The data are not exhaustive but are representative of the range of chemical types and classes that could be present in treated sewage. The data in Table 4.4 are used here to develop and illustrate the approach taken for determining guideline values. The approach can be applied to any chemical of interest.

A2.2 Step 2 — Is there an existing drinking water guideline?

Having identified chemicals of interest, the next step is to determine whether a drinking water guideline has already been set for that chemical. Box A2 lists established drinking water guidelines produced by authorities around the world, as examples of the type of document that can be searched to match against the chemicals of interest. The sources are listed in order of preference of acceptance, based on recommendations from the National Health and Medical Research Council (NHMRC) and the enHealth Council of Australia in relation to risk assessment of environmental hazards (enHealth 2004).

In developing the guideline values given in this document (Table 4.4), the guidelines listed in Box A2 were searched. In line with the recommendations of the NHMRC and enHealth Council, drinking water guidelines from Australia and the World Health Organization (WHO) were given preference over those of other authorities.

The guidelines for chemicals given in the *Australian Drinking Water Guidelines* (ADWG) (NHMRC–NRMMC 2004) are largely based on the methods and outcomes of the relevant WHO publications. However, there are some distinctions between the WHO and Australian drinking water guidelines; for example:

- the WHO guidelines assume a bodyweight of 60 kg, to cater for the lighter bodyweights of developing countries; however, Australian guidelines assume a bodyweight of 70 kg
- for carcinogenic compounds, the WHO guidelines use a risk assessment calculation, with the guideline value set at the concentration that would give rise to a risk of one additional cancer per 100 000 people, whereas the Australian guideline values for these types of compounds are based on a risk of one in a million.

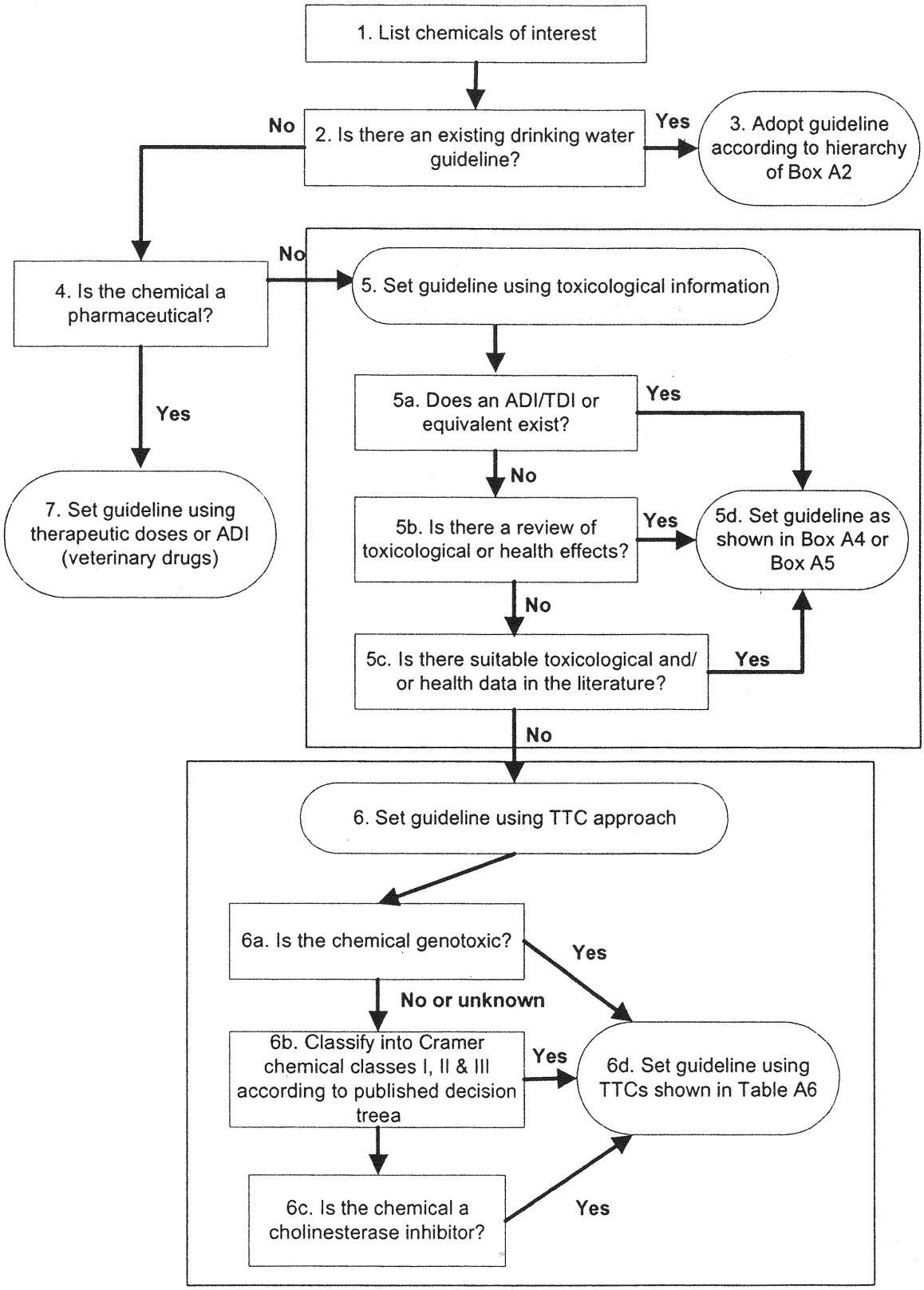
Where WHO guidelines for non-threshold chemicals have been used in this appendix, the values have been adjusted to take into account the lower level of risk used in the Australian guidelines.

Box A2 Hierarchy of drinking water guidelines

The following list details documents in which drinking water guidelines can be found. As described in Section A2.3, it follows a hierarchy, with the *Australian Drinking Water Guidelines* (ADWG) (NHMRC–NRMMC 2004) taking precedence over other publications.

- NHMRC–NRMMC (National Health and Medical Research Council – Natural Resource Management Ministerial Council) (2004). *Australian Drinking Water Guidelines* (ADWG). http://www.nhmrc.gov.au/publications/_files/adwg_11_06.pdf
- WHO (World Health Organization) (2006). *Guidelines for Drinking-Water Quality*, third edition, incorporating first addendum http://www.who.int/water_sanitation_health/dwq/gdwq3rev/en/index.html
- EU (European Union) (1998). *Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption*, Official Journal L 330, 05/12/1998 p 0032–0054. http://ec.europa.eu/environment/water/water-drink/index_en.html
- NZ MoH (New Zealand Ministry of Health) (2005). *Drinking Water Standards for New Zealand*, NZ MoH, Wellington, New Zealand http://www.moh.govt.nz/moh.nsf/0/12F2D7FFADC900A4CC256FAF0007E8A0/$File/drinkingwaterstandardsnz-2005.pdf.
- Health Canada (2006). *Guidelines for Canadian Drinking Water Quality*. http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/doc_sup-appui/index_e.html
- US EPA (United States Environmental Protection Agency) (2007). *Drinking Water Contaminants Lists*. Office of Water, US EPA. http://www.epa.gov/safewater/hfacts.html
- OEHHA (Office of Environmental Health Hazard Assessment) (Various dates). *Public Health Goal for Chemical Substances in Drinking Water*. California Environmental Protection Agency. http://www.oehha.ca.gov/water/phg/allphgs.html
- US EPA (United States Environmental Protection Agency) (Various dates). *Health Advisories for Drinking Water Contaminants*. Office of Water, US EPA.

Figure A1 **Decision tree for setting guidelines for chemicals in recycled water that will be used as a source of drinking water**



ADI = acceptable daily intake; TDI = total daily intake; TTC = threshold of toxicological concern

a Guideline values for chemicals that cannot be classified are calculated using the TTC for genotoxic compounds

Table A7 Cramer classification of compounds without toxicological information that are not genotoxics, pharmaceuticals or cholinesterase inhibitors

Chemical name	Toxtree classification class	TTC (µg/kg bw/day) [a]	Recommended drinking water guideline (µg/L) [b]
Organic compounds			
Musks			
Musk tibetene	III	1.5	0.35 [b]
Pentamethyl-4,6-dinitroindane (Musk moskene)	III	1.5	0.35 [b]
Other compounds			
(Propylenedinitrilo)tetraacetic acid (PDTA)	III	1.5	0.7 [c]
1,7-Dimethylxanthine (Paraxanthine)	III	1.5	0.7 [c]
2,5-Dihydroxybenzoic acid	I	30	7 [b]
2,6-di-tert-butylphenol (2,6-bis(1,1-dimethylethyl)phenol)	II	9	2 [b]
4-Acetyl-6-t-butyl-1,1-dimethylindan	I	30	7 [b]
4-cumylphenol	III	1.5	0.35 [b]
6-Acetyl-1,1,2,4,4,7-hexamethyltetraline	II	9	2 [c]
Bromoacetic acid	III	1.5	0.35 [b]
Bromochloroacetonitrile	III	1.5	0.7 [c]
Caffeine	III	1.5	0.35 [b]
Chlorophene	III	1.5	0.35 [b]
Cholesterol	I	30	7 [b]
Coprostanol (5beta-Cholestan-3beta-ol)	III	1.5	0.7 [c]
Diatrizoate sodium	III	1.5	0.35 [b]
Diatrizoic acid	III	1.5	0.35 [b]
Monobutyltin	III	1.5	0.7 [c]
Triclosan	III	1.5	0.35 [b]
Genotoxic compounds			
2,6-di-tert-butyl-1,4-benzoquinone (2,6-bis(1,1-dimethylethyl)-2,5-Cyclohexadiene-1,4-dione)		0.02	0.14 [c]
5-methyl-1H-benzotriazole		0.02	0.07 [b]
Cholinesterase inhibitors			
Fyrol FR 2 (tri(dichlorisopropyl) phosphate)		0.3	1 [b]
Triphenyl phosphate		0.3	1 [b]
Tris(2-chloroethyl)phosphate		0.3	1 [b]

TTC = threshold of toxicological concern

a Drinking water guidelines taken from Table A6.

b Likely to be in commercial use, P = 10%.

c Presumed not to be in commercial use, P = 20%.

A2.7 Step 7 — Pharmaceuticals

The chemicals listed in Table 4.4 (Chapter 4) include many that are active ingredients of pharmaceutical compounds. In the human body, pharmaceuticals are generally metabolised and cleared as the parent compound and metabolites. Excretion from the body is the primary source of pharmaceuticals in wastewater. Less commonly, pharmaceuticals may be introduced through

industrial accidents and releases from hospitals. Although raw waters may contain limited quantities of pharmaceuticals, it is unusual to find measurable concentrations in drinking water.

A regulatory framework for establishing guidelines for pharmaceutical substances in drinking water was not identified in developing these guidelines. Pharmaceuticals in water present an unusual challenge in choosing the health or toxicological endpoint that underpins the ADI/TDI in Equation 2 of Box A4a. Should the NOEL (or NOAEL) be based on toxicity, as it is for other chemicals in water, or on the biological effect responsible for the pharmacological or therapeutic action?

The TTC approach has not been applied to pharmaceuticals. Active compounds of pharmaceutical products are arguably the most extensively examined chemicals, with clear definitions of toxicity and appropriate pharmacological doses. Because the biological activity (ie the therapeutic effect) of pharmaceuticals is so well defined, it is unusual for TDIs based on toxicity to be established for these chemicals. The exception is for pharmaceuticals used for agricultural and veterinary purposes where TDIs have been established by bodies such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) the Australian Therapeutics Goods Administration (TGA) and the European Agency for the Evaluation of Medicinal Products (EMEA). These TDIs have been used to determine guideline values.

The biological or pharmacological activity at therapeutic doses for pharmaceuticals used for humans is well known, and can be found in the manufacturer's literature and in various pharmacopoeias. The recommended therapeutic doses of pharmaceuticals are intended to elicit a biological outcome (albeit beneficial) in 100% of patients. However, the ratio of doses causing toxicity to the doses giving a beneficial effect (the therapeutic index) is intended to be large.[14] Hence, to establish a drinking water guideline for a pharmaceutical chemical, the approach is to divide the recommended therapeutic dose by a factor that would provide reasonable assurance that effects, either pharmacological or toxic, would be unlikely. Toxicological profiles of pharmaceuticals indicate that none will have a therapeutic or other biological effect at daily doses a hundredfold less than the lowest therapeutic dose. This approach has been applied by Schwab et al (2005) in a human health risk assessment of pharmaceuticals in United States surface waters.

Dolan et al (2005) took a different approach to assessing the risk of pharmaceuticals in environmental media. The authors reviewed ADI values derived since 1981 for active pharmaceutical ingredients of the Merck pharmaceutical company. The analysis excluded genotoxic compounds. The database consisted of 120 compounds, with a broad range of potencies that are administered orally or parenterally. The study found that 94% of the compounds with known pharmacological activity had ADIs[15] greater than 10 µg/day (ie 0.15 µg/kg/day); this ADI applied to Equation 2 of Box A.4a would result in a drinking water guideline of 5 µg/L.

The approach adopted here is to calculate surrogate TDIs (S-TDIs) for pharmaceutical agents by dividing the lowest recommended therapeutic dose (as mg/kg/day) by safety factors, as follows:

- *all pharmaceuticals*— a safety factor of 100 is applied, comprising 10 for differences in response between humans (intraspecies variation) and 10 for the lowest therapeutic dose not being a no-effect level

[14] Many of the pharmaceutical compounds in Table 4.4 are nonsteroidal anti-inflammatory agents, antibiotics, or beta-blockers. These agents would be expected to have a therapeutic index of much more than 10 fold.
[15] Dolan et al (2005) do not provide the basis of the ADIs (ie whether set on pharmacological or toxic NOEL) or the magnitude of the uncertainty factor applied to the NOEL.

- *cytotoxic drugs* — an additional safety factor of 10 is applied due to the higher level of toxicity associated with these compounds
- *hormonally active steroids* —an additional safety factor of 10 is applied on the grounds that their potential effects on hormonal function and fertility is unwanted in those not being treated .

Based on the rational that pharmaceutical chemicals are not widespread in the environment or likely to be present in food, the proportion of the S-TDI allocated to water for pharmaceuticals is 100%. For persons taking medication, intake of a pharmaceutical chemical at the recommended drinking water guideline determined using this methodology (shown in Box A6) will be an additional 1% of their daily dose or 0.1% for cytotoxic drugs or steroidal hormones.

An example of the recommended approach can be seen with norfloxacin, which has been found at concentrations of up to 7 µg/L in wastewater. The lowest recommended daily dose in two parts is 800 mg (ie 400 mg every 12 hours). For a 70 kg adult, that represents a dose of 11.4 mg/kg/day. Applying the above rules, this would mean that water concentrations of norfloxacin should not exceed 4000 µg/L, which is substantially in excess of the measured concentration of 7 µg/L identified in wastewater. A similar process can be applied to any pharmaceutical. Because of the continuous introduction of new pharmaceuticals to the pharmacopoeia, any listing of the lowest doses of pharmaceuticals already available would rapidly lose currency. Given these circumstances, it is better to identify the pharmaceutical agent in the water supply and thereafter apply these principles to the concentrations found. This process will be effective whatever the origin of pharmaceuticals (eg appropriate therapeutic use, hospital discharges or inadvertent releases into water bodies).

Box A6 Calculation of drinking water guidelines using therapeutic doses

$$\text{Drinking water guideline } (\mu g/L) = \frac{\text{S-TDI (mg/kg/day)} \times \text{bw (kg)} \times P \times 10^{-3}}{\text{V (L/day)}} \qquad \text{Equation 4}$$

Where:

S-TDI = surrogate-TDI (mg/kg/day) = LTD ÷ SF (100 or 1000)
P = proportion of S-TDI from water = 100%
bw = bodyweight (70kg)
V = volume of water drunk (2L/day)
10^3 = unit conversion mg/L to µg/L.

If using the lowest therapeutic dose directly instead of the S-TDI, Equation 4 becomes:

$$\text{Drinking water guideline } (\mu g/L) = \frac{\text{LTD (mg/day)} \times P \times 10^{3}}{\text{SF x V (L/day)}} \qquad \text{Equation 4a}$$

Where:

LTD = lowest daily oral therapeutic dose for an adult. The LTD is taken from (in order of priority) MIMS, Martindale, or another pharmacopeia for preparations that have the chemical as a sole ingredient. If dose information is not available for the single agent, then doses from combination preparations are used. If an LTD cannot be located, then either the LTD for a similar active ingredient can be used with an extra safety factor of 10, or a TTC can be derived using a Cramer classification.

SF = safety factor; 100 for most pharmaceuticals, 1000 for cytotoxic compounds and 1000 for synthetic or natural hormones.

Table A8 presents calculated drinking water guidelines for the pharmaceutical chemicals identified in Table 4.4 and compares them with the highest concentrations measured in secondary treated effluent. With limited exceptions the margins of exposure resulting from this comparison are greater than 1 with many being 1000 or more. Given that this does not take into account reductions achieved by advanced treatment processes, it is unlikely that pharmaceutical chemicals will be present at levels approaching the recommended drinking water guideline, or cause untoward effects in people drinking water produced from recycled water.

The exceptions are the estrogenic hormones 17α-ethynylestradiol, estrone and mestranol, the antibiotic chlorotetracycline and the antimetabolite methotrexate. These compounds will be removed by advanced treatment such as reverse osmosis. Concentrations in treated water should be below the calculated guideline values.

Table A8 Recommended drinking water guideline for pharmaceuticals

Pharmaceutical	Highest effluent conc (µg/L)	LTD (mg/day) or ADI (ug/kg/day)[a]	Recommended drinking water guideline (µg/L)	Margin of exposure (DWG ÷ highest conc)
Antibiotics				
Amoxycillin	5	ADI 200 µg/kg/day [b]	7000	1400
Anhydro-erythromycin A	0.92	700	350[c]	380
Azithromycin	0.072	ADI 11 µg/kg/day [o]	40	555
Chloramphenicol	23	3,500	17,500	760
Chlorotetracycline	160	ADI 30 µg/kg/day[g]	105 [c]	0.65
Ciprofloxacin	0.03	500	2,500	83,000
Clarithromycin	0.24	500	2,500	10,400
Clindamycin	0.120	600	3,000	25,000
Demeclocycline	1.12	600	3,000	2,700
Doxycycline	0.03	ADI 3 µg/kg/day [e]	105	3,500
Enrofloxacin	0.002	ADI 6.2 µg/kg/day [e]	220	110,000
Erythromycin	1.7	ADI 5 µg/kg/day [e]	175	103
Lincomycin	0.015	ADI 1000 µg/kg/day [b]	35,000	2,300,000
Monensin	80	ADI 10 µg/kg/day [b]	350	4.4
Naladixic acid	0.22	2,000	10,000	45,500
Norfloxacin	7	800	4,000	570
Penicillin G	0.03	ADI 2.5 µg/kg/day [f]	15	500
Penicillin V	0.21	ADI 2.5 µg/kg/day [f]	15	71
Roxithromycin	460	300	1,500	3.3
Sulfadimethoxine	0.06	500	350	5800
Sulfamethazine	0.22	4000	350	1590
Sulfamethiazole	0.13	1,500	350	2690
Sulfamethoxazole	94	1,600	350	3.7

Pharmaceutical	Highest effluent conc (µg/L)	LTD (mg/day) or ADI (ug/kg/day)[a]	Recommended drinking water guideline (µg/L)	Margin of exposure (DWG ÷ highest conc)
Terramycin (Oxytetracycline)	0.34	ADI 30 µg/kg/day [g]	105 [c]	310
Tetracycline	0.11	ADI 30 µg/kg/day [g]	105 [c]	950
Trimethoprim	0.35	ADI 20 µg/kg/day [b]	700	2000
Tylosin	5	ADI 300 µg/kg/day [b]	10500	2100
Nonsteroidal anti-inflammatory drugs (NSAIDs)				
Aspirin	2.1	ADI 8.3 µg/kg/day [e]	290	120
Diclofenac	0.81	ADI 0.5 µg/kg/day [e]	18	22
Dipyrone	7.5	ADI 150 µg/kg/day [e]	5,250	700
Fenoprofen	0.759	900	4,500	5,900
Ibuprofen	28	800	4,000	142
Indomethacin	0.6	50	250	140
Ketoprofen	0.38	ADI 1 µg/kg/day [b]	35	92
Naproxen	0.57	440	2,200	3,800
Tolfenamic acid	1.6	ADI 5 µg/kg/day [b]	175	110
β-andrenergic blockers				
Betaxolol	0.19	20	100	530
Bisoprolol	0.37	1.25	6.25	17
Carazolol	0.12	ADI 0.1 µg/kg/day [h]	3.5	29
Metoprolol	2.2	50	250	110
Nadolol	0.06	40	200	3,300
Propranolol	0.29	80	400	1,400
Timolol	0.07	20	100	1,400
Estrogenic compounds				
17α-ethinyl estradiol	0.062	0.03	0.015	0.24
17α-estradiol	0.074		0.175j	2.4
17β-estradiol	0.027	ADI 0.05 µg/kg/day [i]	0.175	6.5
Equilenin (horse steroid)	0.278	0.6	0.3	1.1
Equilin	0.15	0.6	0.3	2.0
Estriol	0.051	1	0.5	10
Estrone	0.7	0.6	0.3	0.4
Mestranol	0.407	0.05	0.025	0.06
Norethindrone	0.872	5	2.5	2.9
Progesterone	0.199	ADI 30 µg/kg/day [i]	105	530
Androgenic compounds				

Pharmaceutical	Highest effluent conc (µg/L)	LTD (mg/day) or ADI (ug/kg/day)[a]	Recommended drinking water guideline (µg/L)	Margin of exposure (DWG ÷ highest conc)
Androsterone	0.214	-	14.0[k]	65
Testosterone	0.214	ADI 2 µg/kg/day[i]	7.0	33
Other pharmaceuticals				
Acetaminophen (paracetamol)	4.3	ADI 50 µg/kg/day[e]	1750	410
Alprazolam	0.62	0.5	2.5	4
Antipyrine	0.41	2,000	10,000	24,000
Atorvastatin	0.044	10	50	1,100
Bezafibrate (Benzafibrate)	4.6	600	3,000	650
Carbamazepine	27.3	200	1,000	37
Cefaclor	1.21	500	2,500	2,000
Cephalexin	0.09	ADI 10 µg/kg/day[b]	350	3900
Cimetidine	0.58	400	2,000	3,400
Clenbuterol	0.05	ADI 4.2 µg/kg/day[h]	150	3,000
Clofibric acid (Clofibrate)	1.6	1,500	7,500	4,700
Codeine	9.1	100	500	55
Cotinine ((S)-1-methyl-5-(3-pyridinyl)-2-Pyrrolidinone)[l]	0.9	20[e]	100	110
Cyclophosphamide	0.02	70	35[d]	1,750
Dehydronifedipine[m]	0.03	40	200	6700
Diazepam	2.92	5	25	9
Diltiazem	0.049	120	600	12,200
Enalaprilat	0.046	2.5	12.5	270
Fluoxetine	0.012	20	100	8300
Gemfibrozil	0.42	1,200	6,000	14,300
Iohexol	1.6	1,440	7,200	4,500
Iopamidol	1.6	800	4,000	2,500
Iopromide	1.8	1,500	7,500	4,200
Isophosphamide[n]	2.9	70	35[d]	12
Metformin	0.15	500	2,500	16,700
Methotrexate	1	0.1	0.05[d]	0.05
Salbutamol	0.035	6	30	860
Salicylic acid	2.1	Topical preps only. Cramer class 1	105	50
Stigmastanol	4	2,000	10,000	2500
Sulfasalazine	0.12	1,000	5,000	41,700
Temazepam	1.64	10	50	30
Terbutaline	0.12	9	45	380

a ADI's used for veterinary drugs and where published by EMEA, WHO or TGA
b TGA (2006).
c Similar pharmaceutical, composite safety factor of 1000.
d Cytotoxic, or genotoxic agent, composite safety factor of 1000.
e EMEA (various dates).The European Agency for the Evaluation of Medicinal Products. Veterinary Medicines Evaluation Unit.
f The maximum permitted daily intake of 30 µg parent compound per person (0.43 µg/kg bw/day), is agreed for penicillins in relation to the prevention of allergic reactions (EMEA 2005).
g An ADI of 30 µg/kg bw/day was established for the tetracyclines (oxytetracycline, chlortetracycline and tetracycline) alone or in combination (WHO/JECFA 1998).
h Although an ADI for this compound has been published by the WHO, the EMEA published ADI value has been sourced on the basis that the EMEA report is a more recent evaluation.
i WHO/JECFA 2000
j Assumed same potency as 17β-oestradiol.
k Androsterone is a weak androgen; here it is assumed to be 50% of testosterone potency.
l Cotinine is major metabolite of nicotine, rapidly cleared by the kidneys. Less active than nicotine which is given in antismoking regimes from about 10 mg/person (transdermal). Assume 50% activity of nicotine gives 20 mg/person for cotinine.
m Dihydronifedipine is the predominant metabolite of nifedipine. Minimal dose of nifedipine is 20 mg/day; assume 50% activity for the metabolite yields 40 mg/person.
n Isomer of cyclophosphamide
o Azithromycin is a chemically closely related parent compound of tulathromycin. A toxicological ADI of 11 µg/kg bw/d has been adopted for azithromycin and applies to tulathromycin, based on a 3-month subchronic toxicity study in dogs and rats and a safety factor of 100 (EMEA 2004). An additional safety factor of 10 has been used in the calculation of a DWG on the basis that the ADI from a closely related compound (tulathromycin) was used.
p A guideline for sulphonamides in drinking water made from recycled water has been established herein by applying the lowest ADI for sulphonamides established by the NRA (i.e. 0.1 mg/kg bw/d [NRA 2000]). This yields a DWG of 350 µg/L. It is recommended that this be applied to all individual sulphonamides.

A3 Validation of the threshold of toxicological concern for drinking water standards

To assess the validity of the Cramer class NOELs as assigned by Munro et al (1996) and others for use in setting drinking water standards, organic compounds for which there is a drinking water standard (from NHRC 2004 and WHO 2006) have been classified into the three Cramer classes using ToxTree. The following analysis was then undertaken:

1. The cumulative frequency of drinking water guideline for each of the Cramer classes was compared with the drinking water guideline established using the TTC for the classes (Figure A2).

2. Cumulative distributions of safety factors from the ADWG (NHMRC–NRMMC 2004) and WHO guidelines (2006) were applied to organic compounds when setting drinking water guidelines (Figure A3).

3. The frequency distribution of the known NOELs (used to set the drinking water standard) was compared to the NOELs for the same compounds in the Munro et al (1996) databases (Figure A4).

4. Compounds that have a drinking water guideline NOEL, and also a NOEL in the Munro database, were classified using ToxTree. The cumulative distributions of the Munro NOELs for Cramer classes I and III were then compared with the cumulative distributions of NOELs from the drinking water guideline database (Figure A5).

Letter

Pharmaceuticals in drinking water: is the cure worse than the disease?

Australia is discussing the draft report *Guidelines for Water Recycling* (*1*). The inclusion of pharmaceutical ingredients represents the first attempt by a national authority to set guidelines for levels of these emerging micropollutants in drinking water. Tolerable daily intakes for all pharmaceuticals have been calculated by dividing the lowest recommended therapeutic dose (LRTD) by a safety factor of 100, comprising 10 for intrahuman variability and 10 for the LRTD not being a no-effect level. An additional safety factor of 10 has been applied to cytotoxic and endocrine-active compounds. This is equivalent to declaring that 1% of the therapeutic dose for all drugs, or 0.1% for cytotoxins and hormones, is to be considered harmless in a chronic-exposure scenario. Therapeutic doses are not "real" toxicity data; they are derived from less-than-lifetime studies, and they give no indication of toxicity threshold levels. The risk assessment safety factor of 10 used for extrapolation of animal data to humans generally accounts for potentially more susceptible human groups and is not considered in the draft guidelines. In addition, drugs in the environment occur in mixtures. Given these uncertainties and the absence of any established guideline, a comprehensive and more conservative safety factor of at least 100,000 should be adopted for all pharmaceuticals.

An increasing number of scientists are concerned about pharmaceuticals in drinking water. It is crucial to be more conservative on a key, emerging issue where uncertainty is the highest, so as not to discredit a genuine guideline-setting approach in the eyes of the community. I am convinced that we should give the community in Australia and the world a strong message of no compromise with regard to the protection of human health in matters of primary resources such as drinking water.

FRANCESCO POMATI
University of New South Wales
Sydney, Australia
f.pomati@unsw.edu.au

(1) Australian Environment Protection and Heritage Council. *Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (Phase 2): Augmentation of Drinking Water Supplies*; www.ephc.gov.au/ephc/water_recycling.html.

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑　誌

著者氏名	論文タイトル名	発表誌名	号	ページ	年
島崎大、西村哲治、国包章一	水道水源等の医薬品による汚染とその制御	かんきょう	1	26-27	2007
Hirabayashi Y, Yoon BI, Tsuboi I, Huo Y, Kodama Y, Kanno J, Ott T, Trosko JE, Inoue T	Protective role of connexin 32 in steady-state hematopoiesis, regeneration state, and leukemogenesis	Exp Biol Med	232	700-712	2007
Minami A, Tsuboi I, Harada T, Fukumoto T, Hiramato M, Koshinaga M, Hirabayashi Y, Kannno J, Inoue T, Aizawa S	Inflammatory biomarker, neopterin, suppresses B lymphopoiesis for possible facilitation of granulocyte responses, which is severely altered in age-related stromal-cell-impaired mice, SCI/SAM.	Exp Biol Med	232	134-145	2007
Hirabayashi Y, Inoue T	Implication of hempoopoietic progenitor cell kinetics and experimental leukemogenesis: relevance to Gompertzean mortality as possible hepatotoxicological endpoint	Exp Hematol	35	125-133	2007
Hirabayashi Y, Yoon BI, Tsuboi I, Huo Y, Kodama Y, Kanno J, Ott T, Trosko JE, Inoue T	Membrane Channel Connexin 32 Maintains Lin^{-}/c-kit^{+} Hematopoietic Progenitor Cell Compartment : Analysis of the Cell Cycle	J Membr Biol	217	105-113	2007
Yoshida K, Hirabayashi Y, Wada S, Watanabe F, Watanabe K, Aizawa S, Inoue T	p53(TRP53) Deficiency-Mediated Antiapoptosis Escape after 5 Gy X Irradiation Still Induces Stem Cell Leukemia in C3H/He Mice: Comparison between Whole-Body Assay and Bone Marrow Transplantation(BMT) Asaay	Radiat Res.	167	703-710	2007
Vermeire T, Munns WRJr, Sekizawa J, Suter G, Kraak GV	An Assessment of Integranted Risk Assessment	Hum Ecol Risk Assess	13	339-354	2007
関澤　純	内分泌かく乱化学物質による低用量影響の蓋然性	日本リスク研究学会誌	17	79-84	2007
Nakamura Y, Yamamoto H, Sekizawa J, Kondo T, Hirai N, Tatarazako N	The effect of pH on fluoxetine in Japanese medaka (*Oryzias latipes*) :Acute toxicity in fish larvae and bioaccumulation in juvenile fish	Chemosphere	70	865-873	2008
田村生弥、太田美菜子、関澤　純、山本裕史	下水道未普及地域における河川生物膜による直鎖アルキルベンゼンスルホン酸浄化作用の評価	Enviromental Engineering Research	44	127-134	2007
Yamamoto H, Nakamura Y, Nakamura Y, Kitani C, Imari T, Sekizawa J, Takao Y, Yamashita N, Hirai N, Oda S,Tatarazako N	Initial Ecological Risk Assessment of Eight Selected Human Pharmaceuticals in Japan	Environmental Sciences	14	177-193	2007
山本裕史、中村友紀、木谷智世、中村雄大、関澤　純、鑪迫典久	非ステロイド系医薬品類の生態リスク評価	Environ Sanit Eng Res	21	71-78	2007

水道水源等の医薬品による汚染とその制御

島崎 大
西村 哲治
国包 章一
厚生労働省国立保健医療科学院水道工学部
厚生労働省国立医薬品食品衛生研究所環境衛生化学部
厚生労働省国立保健医療科学院水道工学部

1 はじめに

欧米諸国では1990年代半ばから、また国内では主に2000年以降から、医療用や畜産用の医薬品及びその代謝物を起源とする生理活性や薬理活性を有する化学物質が、下水処理水や河川水から検出されています。これら化学物質の環境中における存在濃度は極わずかであると考えられますが、ヒトの神経系や代謝系などの恒常性に影響を及ぼす物質が含まれるため、その挙動や影響について関心が寄せられています。筆者らは、排水等を通じて水道水源に流出し、また残留する可能性のある医薬品が水道水に及ぼす影響について主眼を置き、標記の調査研究を行っています。ここでは、これまでに得られた研究成果について概要を紹介します。

2 優先して監視すべき医薬品の選定

現在、国内で医薬品原体として流通している化学物質は数千種にのぼるため、各種の排水等を通じて水環境中に流出しやすい物質を選定して調査対象とする必要があります。まず、これまでに水環境中での検出実績のある物質を中心に120種を選定し、さらに各物質の生産量や物性情報に基づいた優先度の評価を行いました。評価項目は、生産量、尿中排泄率、オクタノール／水分配係数（Kow）、生分解性指標値、検出濃度／1日最小用量の5項目として、各項目を5段階にスコア化した合計値を比較しました。合計値が上位である物質を挙げると、解熱鎮痛剤のアスピリン、アセトアミノフェン、サリチルアミド、ジクロフェナク、スルピリン、抗菌剤のレボフロキサシン、テトラサイクリン、クラリスロマイシン、スルファメトキサゾール、硫酸カナマイシン、硫酸ストレプトマイシン、X線造影剤のイオパミドール、イオプロミド、イオメプロール、抗てんかん剤のカルバマゼピン、バルプロ酸ナトリウム、フェニトイン、鎮咳剤のテオフィリン、塩酸エフェドリン、高脂血症治療剤のベザフィブラート、高血圧治療剤の塩酸ヒドララジン、精神情動安定剤のスルピリド等です。この中の半数以上の医薬品は、これまでに国内外の水環境中での調査対象となっていますが、特に水に溶けやすい医薬品については適当な分析法が確立しておらず、その存在が明らかでない状況です。今後は調査事例がない物質についても、分析法を含めた検討を進める必要があると考えています。

3 水道原水や排水等の医薬品による汚染実態調査

これまでにLC／MS／MS法、LC／MS法及び誘導体化GC／MS法による分析法を検討し、平成17年度は河川水（3河川、23地点）、下水処理場流入水及び放流水（6処理場）、浄水場原水及び浄水（6浄水場）を対象とした医薬品の実態調査を行いました。河川水中から検出された医薬品は26種類にのぼりました。特に多摩川での存在濃度や検出頻度が高い傾向にあり、ベザフィブ

ラート（高脂血症治療剤）やメフェナム酸（解熱鎮痛剤）が最大1μg／L前後で検出されました。ただし、検出された地点はいずれも中流域であり、上流域では全く検出されなかったため、これらは下水由来であると推定されます。また下水処理場への流入水を対象とした調査では、実際に36種類の医薬品が高濃度で検出されており、例としてサリチル酸（解熱鎮痛剤）、アセトアミノフェン（同上）、ベザフィブラートの3物質は最大濃度が10μg／Lを超えました。下水放流水では、ほとんどの医薬品は流入水よりも低い濃度であり、1μg／Lを超えて検出されたのはベザフィブラート、ジフェンヒドラミン（抗アレルギー薬）、トリクロサン（抗菌剤）でした。さらに、水道原水からも20種類の医薬品が0・001～0・1μg／Lの範囲で検出されましたが、浄水からはほとんど検出されませんでした。ただし浄水場での調査はスポット採水1回のみであるため、今後も継続調査が必要です。

4　浄水処理による制御方法

浄水処理による医薬品類の除去性に関しては、オゾン処理や膜ろ過等の知見はあるものの、他の処理方式の情報は限られている状況です。そこで、基本的な処理である凝集沈殿及び塩素処理、また農薬等の微量化学物質の除去に用いられている粉末活性炭処理について検討を行いました。対象とした医薬品は、解熱鎮痛剤のイブプロフェン、ケトプロフェン、ナプロキセン、ジクロフェナク、インドメタシン、フェノプロフェン、プロピフェナゾン、高脂血症治療剤のゲンフィブロジル、高脂血症治療剤の代謝物であるクロフィブリック酸の9物質です。医薬品の初期濃度は各100μg／Lとしました。凝集沈殿処理では、凝集剤としてポリ塩化アルミニウムを用いたジャーテストを行ったところ、模擬濁質であるカオリンが十分に除去されている条件下でも、これらの比較的疎水性が高い医薬品はほとんど除去されませんでした。したがって、他の親水性が高い医薬品についても凝集沈澱処理による除去は困難であると推測されます。塩素処理として、初期有効塩素濃度1・0mg／Lの条件で最大24時間反応させたところ、インドメタシン及びプロピフェナゾンは1時間後に検出されなくなりましたが、ナプロキセン及びジクロフェナクは24時間後の残存率が30％程度、その他の5物質は24時間後も80％以上残存しました。一部の医薬品は速やかに塩素と反応することが示されましたが、何らかの分解物が生成される可能性があるため、分解物の同定やその生理活性の解明が課題です。粉末活性炭処理では、注入率を10mg／Lとした場合に接触1時間後には各医薬品とも83～100％除去されました。ただし医薬品9種を同濃度で混合した系では、特にイブプロフェンとクロフィブリック酸の除去率が競合吸着によって低下し、接触3時間後の除去率は各60％前後となりました。また、実験条件は異なりますが、実際の河川水を使用した場合には溶存有機物等の影響を受けて除去率が半減することが示されました。今回検討した医薬品の一部には、各浄水処理での除去性がいずれも低い物質が存在するため、今後は実際の浄水処理工程での消長についても調査を進める予定です。

5　おわりに

本研究は、環境省地球環境保全等試験研究費の研究課題「水道水源等における生理活性物質の測定と制御に関する研究」として、厚生労働省健康局水道課、国立保健医療科学院、国立医薬品食品衛生研究所の共同で平成16～18年度にかけて実施しております。また、本研究には、北海道大学大学院、東京都健康安全研究センター多摩支所及び各地の水道事業体にご協力いただいております。この場を借りて厚く御礼申し上げます。

Protective Role of Connexin 32 in Steady-State Hematopoiesis, Regeneration State, and Leukemogenesis

YOKO HIRABAYASHI,* BYUNG-IL YOON,*[,1] ISAO TSUBOI,* YAN HUO,* YUKIO KODAMA,* JUN KANNO,* THOMAS OTT,† JAMES E. TROSKO,‡ AND TOHRU INOUE§[,2]

*Division of Cellular and Molecular Toxicology, Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo 158-8501, Japan; †Service Einrichtung Transgene Tiere, Hertie-Institut für Klinische Hirnforschung, Tübingen 72076 Germany; ‡Department of Pediatrics and Human Development, Michigan State University, College of Human Medicine, East Lansing, Michigan 48824; and §Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo 158-8501, Japan

The role of gap junctions formed by connexins (Cxs) has been implicated in the homeostatic regulation of multicellular systems. Primitive hematopoietic progenitor cells form a multicellular system, but a previous report states that Cx32 is not expressed in the bone marrow. Thus, a question arises as to why Cx molecules are not detected in the hematopoietic tissue other than in stromal cells. Based on our preliminary study, which suggested a potential impairment of hematopoiesis in Cx32-knockout (KO) mice, the objectives of the present study were to determine whether Cx32 functions in the bone marrow during steady-state hematopoiesis and to examine its possible protective roles during regeneration after chemical abrasions and during leukemogenesis after the administration of a secondary genotoxic chemical, methyl nitrosourea (MNU). As a result, the Cx32 molecule, functioning in the hematopoietic stem cell (HSC) compartment during steady-state hematopoiesis, was observed for the first time; the expressions of Cx32 at the mRNA level, as determined by polymerase chain reaction analysis, and at the protein level, determined using an anti-Cx32 antibody, were observed only in the lin$^-$c-kit$^+$ HSC fraction, using a combination of immunobead-density gradient and immunomagnetic bead separation. Hematopoiesis was impaired in the absence of Cx32, and it was delayed during regeneration after chemical abrasion with 5-fluorouracil at 150 mg/kg body wt in Cx32-KO mice. Cx32-KO mice showed increased leukemogenicity compared with wild-type mice after MNU injection; furthermore, in a competitive assay for leukemogenicity in mice that had been lethally irradiated and repopulated with a mixed population of bone marrow cells from Cx32-KO mice and wild-type mice, the resulting leukemias originated predominantly from Cx32-KO bone marrow cells. In summary, the role of Cx32 in hematopoiesis was not previously recognized, and Cx32 was expressed only in HSCs and their progenitor cells. The results indicate that Cx32 in wild-type mice protects HSCs from chemical abrasion and leukemogenic impacts. Exp Biol Med 232:700–712, 2007

Key words: connexin 32 (Cx32); hematopoietic stem cell; Cx32-knockout mouse; tumor suppressor; experimental leukemogenesis

Introduction

Connexin (Cx) functions in the organization of cell-cell communication *via* gap junctions in multicellular organisms. Gap junctions have been implicated in the homeostatic regulation of various cellular functions, including growth control and differentiation (1), apoptosis (2), and the synchronization of electrotonic and metabolic functions (3).

Radiation exposure and acute tissue injury induce the disconnection of Cxs, resulting in tissue damage (4). On the other hand, the disconnection of Cxs during acute-phase cellular injury also seems to be a protective response that results in active tissue proliferation and consequent recovery. However, transgenic mice expressing a dominant-negative mutant of Cx32 show a notably delayed recovery after partial hepatectomy compared with wild-type mice (5), which implies that the downmodulation of Cx32 is not always advantageous for tissue recovery, despite the

This study was supported by a Grant-in-Aid for Scientific Research B, 11694334, and also by the Japanese Society for the Promotion of Science (JSPS) Invitation Fellowship for Research in Japan, S01275.

[1] Current address: Department of Veterinary Medicine, College of Animal Resource, Kangwon National University, Chuncheon, 200-701, Republic of Korea.

[2] To whom correspondence should be addressed at 1-18-1 Kamiyohga, Setagayaku, Tokyo 158-8501, Japan;. E-mail: tohru@nihs.go.jp

Received July 15, 2006.
Accepted December 7, 2006.

1535-3702/07/2325-0700$15.00

finding that a lack of gap junctional restriction seems to enhance cell proliferation (6) (see also Ref. 7 for current information).

Gap junctions are downmodulated during an acute exposure to promoter chemicals, the carcinogenic relevance of which is as yet not clearly understood (8). Temme *et al.* found that not only spontaneous hepatic tumors but also diethyl-nitrosamine–induced tumors are preferentially induced in Cx32-knockout (KO) mice compared with wild-type mice (9). Why does the downmodulation of Cxs attenuate the protection from malignancy? The reason is that the downmodulation of Cxs results in individual potentially transformable initiated cells that are undergoing independent and infinite growth without interference from surrounding cells; thus, the downmodulation of Cxs in this case seems unlikely to play a protective role (6). On the other hand, the downmodulation of Cxs after exposure to a possible carcinogenic chemical, cadmium, induces cells to undergo apoptosis, which appears to be a protective role (10), though not all cells undergo apoptosis, unfortunately.

The role of Cxs in hematopoietic organs is poorly understood, except in that the expression of Cx43 between hematopoietic progenitor cells and bone marrow stromal cells sustains hematopoiesis (11–14). As Cxs are essential molecules for multicellular organisms, Cxs that organize cell-cell communication within the hematopoietic progenitor cell compartment are surmised to be present in the bone marrow tissue. Recently, we have observed a functional impairment of the bone marrow in Cx32-KO mice in our benzene exposure experiment (15). Krenacs and Rosendaal previously reported that Cx32 is not expressed in the bone marrow (16). If Cx32 is expressed, such Cx32-expressing cells are likely to be rare; for instance, solely in hematopoietic stem/progenitor cells. Hence, similarly to the case of transforming growth factor-β expression, which is observed only in an immature progenitor cell compartment of the bone marrow (17, 18), it seems to be worth studying the expression of Cx32 in the hematopoietic system, particularly in hematopoietic stem/progenitor cells. In this study, we determined whether Cx32 functions solely in primitive hematopoietic cells in a steady-state bone marrow to elucidate its potential protective role during regeneration after bone marrow abrasion and during leukemogenesis after the administration of a secondary genotoxic chemical, methylnitrosourea (MNU).

Cx32-KO mice, first established in 1997 by Nelles *et al.*, can be used for the analysis of the function of Cx32 using a reverse biologic approach (19). In using these mice, the contribution of Cx32, not only in steady-state hematopoiesis and regenerating hematopoiesis but also in the prevention/suppression of leukemogenesis, was elucidated.

Materials and Methods

Experimental Animals. Cx32-KO mice ($Cx32^{-/-}$ or $Cx32^{-/Y}$) were genetically modified from the F1 embryonic cell line, 129/J, and the C57BL/6 strain by K. Willecke (19), who kindly provided these Cx32-KO mice, which were backcrossed with the C57BL/6 strain, and maintained as heterozygous mice ($Cx32^{-/+}$) at the animal facility of the National Institute of Health Sciences (NIHS), Japan. Because the *Cx32* gene is X chromosome linked, male mice carrying the homozygous knockout genotype ($Cx32^{-/Y}$) were generated by mating heterozygous females ($Cx32^{-/+}$) with wild-type males ($Cx32^{+/Y}$). The pups were genotyped by polymerase chain reaction (PCR) screening of DNA obtained from their tails.

Eight-week-old C57BL/6 female mice from Japan SLC (Hamamatsu, Japan) were used as the recipients of bone marrow transplantation. All experimental protocols involving laboratory mice in this study were reviewed by an externally established peer review panel, the Committee of the Ethics of the Research and Welfare of the Experimental Animals of the NIHS, and thereby approved by the Animal Care and Use Committee at the NIHS with the experimental code 224-37009639415-2002. Approved experiments were humanely performed in strict accordance with Guidelines for the Care and Use of Laboratory Animals, NIHS, Japan.

Blood and Bone Marrow Separation. Peripheral blood was collected from the orbital sinus. The numbers of peripheral white blood cells, platelets, and red blood cells were measured using a Coulter counter (Sysmex K-4500; Sysmex Co., Kobe, Japan). Bone marrow cells were harvested from the femur of each mouse (20) after animals were sacrificed by cervical dislocation under deep anesthesia with ethyl ether. A 26-gauge needle was inserted into the femoral bone cavity through the proximal and distal ends of the bone shafts, and bone marrow cells were flushed out under pressure by injecting 2 ml α-minimum essential medium (MEM) with ribonucleosides and deoxyribonucleosides (Invitrogen Corp., Carlsbad, CA). A single-cell suspension was obtained by gently and repeatedly drawing bone marrow cells through a 26-gauge needle and then a 27-gauge needle.

Antibodies. For immunobead-density gradient separation, the biotinylated antibody cocktail (BD Biosciences, San Jose, CA) containing anti-mouse CD3e (145–2C11), CD11b (M1/70), CD45R/B220 (RA3-6B2), Ly-6G and Ly-6C/Gr-1 (RB6-8C5), and TER-119/erythroid cell (TER-119) antibodies; and the monoclonal antibody cocktail SpinSep (StemCell Technologies Inc., Vancouver, BC, Canada) containing anti-CD5/Ly-1, CD45R, CD11b/Mac-1, Ly-6G/Gr-1, TER119, and 7/4/neutrophil antibodies were used as lineage (lin) markers. As a secondary antibody for the former biotinylated antibody cocktail, streptavidin–peridinin chlorophyll, a protein (PerCP; BD Biosciences) was used. For the latter cocktail, SpinSep, an optimized combination antibody cocktail against SpinSep that had been coated on dense microparticles, SpinSep Mouse Dense Particles (StemCell Technologies Inc.), was used for immunoprecipitation.

For immunomagnetic bead separation, CD117/c-kit

conjugated with phycoerythrin (PE; StemCell Technologies Inc.) was used as a progenitor marker, and an anti-PE tetrameric antibody complex (StemCell Technologies Inc.) was used as secondary antibody.

For flow cytometric analyses, the same antibody cocktails from BD Biosciences were used as lineage markers. In addition, a mouse anti-Cx32 monoclonal antibody from two sources (Chemicon International Inc., Temecula, CA, and Santa Cruz Technology Inc., Santa Cruz, CA) was used as the primary antibody for Cx32. As a secondary antibody, anti-mouse Ig conjugated with fluorescein isothiocyanate (FITC; BD Biosciences) was used.

For immunohistochemical analysis, the same anti-Cx32 antibody (Chemicon International, Inc.) was used as the primary antibody. As the secondary antibody, a biotinylated horse anti-mouse IgG antibody (Vector Laboratories Inc., Burlingame, CA) was used, and streptavidin labeled with peroxidase and 3,3′-diamino-benzidine (DAB) was used to detect immunoreactivity (Vector Laboratories Inc.).

Enrichment of Bone Marrow Cells in lin$^-$c-kit$^+$ Fraction. The lin$^-$c-kit$^+$ fraction is rich in hematopoietic stem cells (HSCs). To obtain a large number of lin$^-$c-kit$^+$-enriched fraction in the bone marrow cells, preseparation was carried out by the combination of immunobead density gradient and immunomagnetic bead separation. First, for the depletion of lineage-positive bone marrow cells, harvested bone marrow cells were processed through an immunobead density gradient using a density-matched medium and dense microparticles coated with a cocktail of an optimized combination of antibodies, SpinSep. Second, for selection of the c-kit$^+$ fraction, immunomagnetic bead separation using magnetic nanoparticles with a magnetic holder was carried out using the manufacturer's instruction (StemCell Technologies Inc.). For each procedure, the antibodies used are described in the subsection *Antibodies* in *Materials and Methods*.

Flow Cytometric Analysis Using Anti-Cx32 Antibody. Bone marrow cells with or without fractionation for lin$^-$c-kit$^+$ HSC enrichment were stained with the biotinylated antibody cocktail for streptavidin-PerCP, c-kit–PE, the anti-Cx32 antibody, and anti-mouse IgG conjugated with FITC. For exposure to the intracytoplasmic epitope of the anti-Cx32 antibody, cells were fixed with paraformaldehyde and then permeabilized with phosphate-buffered saline supplemented with HEPES and saponin (21). Flow cytometric analysis was carried out using FACS Vantage (BD Biosciences).

Irradiation. In the assay of hematopoietic progenitor cells, as well as in the repopulation bioassay for leukemogenesis (22), recipient mice were exposed to a lethal radiation dose of 915 cGy at a dose rate of 124 cGy/min using a ^{137}Cs-gamma irradiator (Gammacell 40 Exactor; MDS Nordin Inc., Ottawa, ON, Canada) with a 0.5-mm aluminum-copper filter.

Assay for Colony-Forming Units in Spleen (CFU-S). The Till and McCulloch method was used to determine the number of hematopoietic spleen colonies (CFU-Ss) (23) formed by hematopoietic progenitor cells. Aliquots of a bone marrow cell suspension were used for evaluating the number of CFU-Ss. Spleens were harvested 9 days after the bone marrow transplantation to determine the number of CFU-S-9 and 13 days to determine the number of CFU-S-13, and then were fixed in Bouin solution. Macroscopic spleen colonies were counted under an inverted microscope at magnification ×5.6. It was previously shown using the C57BL/6 strain that all colonies visible on Day 9 and Day 13 originate from the transplanted bone marrow cells under the condition that the recipient mice were exposed to a lethal radiation dose of 915 cGy (24).

Assay for Granulocyte-Macrophage Colony-Forming Units (CFU-GMs). CFU-GMs were assayed in semisolid methylcellulose culture (20, 24). Briefly, 8×10^4 bone marrow cells suspended in 100 μl α-MEM were added to 3.9 ml culture medium containing 1% methylcellulose (Nakarai-Tesque Co. Ltd., Kyoto, Japan), 30% fetal calf serum (HyClone Laboratories Inc., Logan, UT), 1% bovine serum albumin (Sigma, St. Louis, MO), 10^{-4} *M* mercaptoethanol (Sigma), and 10 ng/ml murine granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems Inc., Minneapolis, MN). One-milliliter aliquots containing 2×10^4 cells were placed in 35-mm tissue culture wells (Nalgen Nunc International, Rochester, NY) in triplicate, and were incubated for 6 days in a fully humidified incubator at 37°C with 5% CO_2 in air. Colonies were counted using an inverted microscope at magnification ×40 (Olympus Optical Co. Ltd., Tokyo, Japan).

PCR Analysis for Genotyping. To detect Cx32 wild-type and Cx32-KO alleles, PCR analysis was performed using genomic DNA extracted from the tail of each mouse or from the hematopoietic tissues, spleen and bone marrow, or from tumor cells of the mice in the carcinogenesis tests, and synthetic oligonucleotides were used as primers (19). Hepatic tissues were assayed as the positive control materials (19). To detect the wild-type allele, the common 5′ primer (ccataagtcaggtgtaaaggagc) and the 3′ primer (agataagctgcagggaccatagg) were used; to detect the Cx32-KO allele, the common 5′ primer and *neo*-primer (atcatgcgaaacgatcctcatcc) were used.

Reverse Transcription (RT) and PCR Analysis of Cx32 Expression. The expression of the gene encoding Cx32 was analyzed by RT followed by PCR. The total RNA from the bone marrow cells and other tissues was isolated using a Qiagen RNAeasy kit (Qiagen, Valencia, CA). Since hepatocytes are known to express Cx32 (19), the liver was used not only as the hematopoietic organ, but also as the positive control in the verification by RT-PCR analysis. RT was performed using total RNA with random hexamers as primers, according to the instructions provided with the RT kit from Applied Biosystems (Foster City, CA). PCR amplification was performed using the following previously designed oligonucleotide primers including β-actin primers, an amplification control for RT-