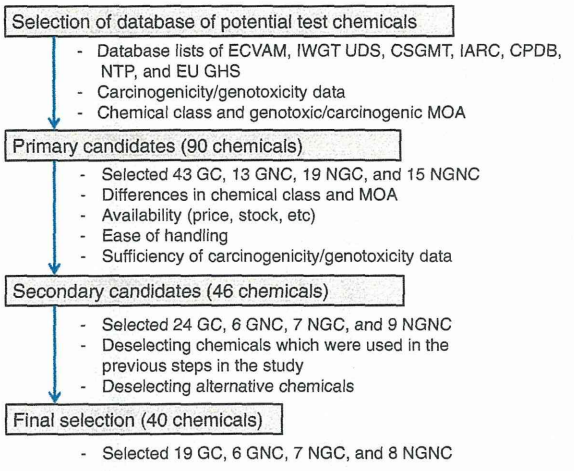


Fig. 1. Flow chart of the different steps of the chemical selection.
Database lists, EVCAM [9], IWGT UDS [10], CSGMT [11], IARC [12], CPDB [13], NTP [14], and *EU GHS* [15].
GC, genotoxic carcinogens; GNC, genotoxic non-carcinogens; NGC, Non-genotoxic carcinogens; NGNC, non-genotoxic non-carcinogens; MOA, mode of actions.

natives were ganciclovir for azidothymidine (AZT), daunomycin hydrochloride and busulfan for mitomycin C (MMC). AZT, a nucleoside analog, and MMC, a DNA-interstrand crosslinker, are both well known carcinogens but are relatively expensive. Among the 43 chemicals, 33 were Ames-positive, while the remaining 10 chemicals were Ames-negative (including one equivocal), but were positive in an *in vitro* CA and rodent *in vivo* erythrocyte micronucleus tests. The majority of the chemicals (37/42) were positive in an *in vitro* CA test; one chemical had no CA data. With respect to the rat liver UDS assay, 13 chemicals were positive, 12 were negative and 1 was inconclusive; there were no UDS data on the remaining 17 chemicals.

• Genotoxic non-carcinogens (13 chemicals)

Thirteen chemicals were selected as candidate genotoxic non-carcinogens. Of these, 11 were Ames-positive, while the remaining 2 chemicals were Ames-negative but were positive in the *in vitro* CA test and rodent *in vivo* micronucleus tests. The majority of the chemicals (11/12) were positive in an *in vitro* CA test, one chemical had no CA data. For the results of liver UDS assay, 1 chemical was positive, 2 were negative, and 1 was inconclusive; there were no UDS data on the remaining 9.

• Non-genotoxic carcinogens (19 chemicals)

Nineteen chemicals were selected as candidate non-genotoxic carcinogens. All chemicals were Ames-negative, except one chemical that was equivocal. For the *in vivo* erythrocyte micronucleus test, 13 chemicals were negative, 4 were inconclusive, and 2 had no micronucleus data. Some chemicals (7/19) were positive in the *in vitro* CA test. For the results of liver UDS assay, 8 were negative and the remaining 11 had no UDS data. Chloroform, ethanol, and methyl carbamate were included as specific non-genotoxic liver carcinogens.

• Non-genotoxic non-carcinogens (15 chemicals)

Fifteen chemicals were selected as candidate non-genotoxic, non-carcinogens. All chemicals were Ames-negative and 10 chemicals were also negative in the *in vivo* micronucleus assay. Some chemicals (5/15) were positive in the *in vitro* CA test. For the results of liver UDS assay, 2 were negative and the remaining 13 had no UDS data. Sodium chloride was included as a specific non-genotoxic, non-carcinogenic gastrotoxicant.

• Positive control (1 chemical)

Ethyl methansulfonate, a genotoxic carcinogen, was used as the concurrent positive control throughout the comet assay validation study.

3.3. Secondary candidate chemicals (46 chemicals excluding positive control)

Forty-six chemicals were selected as secondary candidates from the 90 primary candidate chemicals, based on differences in chemical properties or availability and price (Table 2). Based on the experimental design for the validation study (maximum dose level of 2000 mg/kg *x* number of dose levels (3) *x* number of treatments (4) *x* numbers of rats per dose group (5) *x* expected average rat weight (200 g)), it was estimated that a minimum of 20 g would be needed per study, in the absence of animal toxicity. Therefore, due to budgetary limitations, chemicals with a purchase price of more than 10,000 JPY (equivalent to approximately 100 US $) per gram were generally excluded. Other chemicals were excluded from further consideration because there was little information on their carcinogenicity and/or genotoxicity, they could not be obtained commercially, they were commercially available but the supply was too limited, and/or could not easily be administered orally (Table 2). Where there were multiple chemicals with similar properties (*e.g.*, chemical class, genotoxic mode of action), only one chemical was selected. Based on these criteria, 16 chemicals were excluded on the basis of costs, 2 due to anticipated difficulties in administrating orally, 16 due to similarity in properties, 7 because of limited information on carcinogenicity and/or genotoxicity, and 5 due to lack or limited commercial availability (Table 2).

3.4. Final selection of 40 test chemicals excluding positive control

Forty chemicals were selected as the final reference chemicals from the 46 secondary candidates' chemical list (Tables 2 and 3). The six excluded chemicals included 5 genotoxic carcinogens: acrylamide (due to its use in Phase 2 of the pre-validation study), *N*-methyl-*N*-nitrosourea (due to its use in Phase 3 and Phase 4 – step 1 in the validation study), MMC (due to its cost) although busulfan was used as an alternative, daunomycin hydrochloride, and ganciclovir (an alternative of AZT that was not used), as well as the non-genotoxic non-carcinogen D-mannitol, which was used in Phase 3 and Phase 4 – step 1 in the validation study. Although 2,4-diaminotoluene (genotoxic carcinogen) and 2,6-diaminotoluene (genotoxic non-carcinogen) were used in Phase 2 of the pre-validation study, both chemicals were used also in the main validation study to review inter-laboratory reproducibility. The final 40 reference chemicals included 19 genotoxic carcinogens, 6 genotoxic non-carcinogens, 7 non-genotoxic carcinogens and 8 non-genotoxic non-carcinogens. These were as follows:

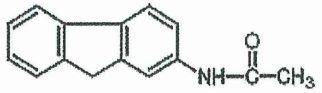
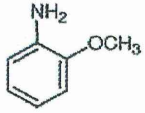
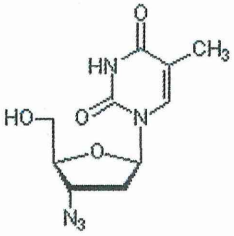
• Genotoxic carcinogens (19 chemicals)

√ 2-Acetylaminofluorene (2-AAF) [Chemical Abstracts Services Registry Number [CASRN] 53-96-3]

Carcinogenicity: IARC [12], Not listed; CPDB [13], positive

2-AAF induces liver tumors in rats and mice, and mammary gland and skin tumors in rats [13]. It is positive in the Ames mutagenicity and *in vitro* CA tests [9,14] and in several *in vivo* genotoxicity tests including MN [20], transgenic (TG) mutation [21,22] and UDS [10,23] assays in rats and/or mice. Metabolic activation is

Table 3
Detailed *in vivo* genotoxicity data on selected final test chemicals for international validation study on the *in vivo* comet assay.

No.	Chemical [CAS] <Carcinogenicity>	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Refs.
	Genotoxic carcinogens (19)							
1	2-Acetylaminofluorene [53-96-3]		MN	+	Rat	po	125–500 × 2d	[20]
			TG (liver)	+	BigBlue mouse	diet	72 × 28d	[21,22]
	<IARC, Not listed; CPDB, +ve>		UDS	+	Rat	po	5, 50	[10,23]
			In vitro Ames/CA	+/+				[9,14]
2	Acrylonitrile [107-13-1]		MN	–	Rat	po	10–40	[11]
			MN	+	Rat	iv	24.5–98	[11]
	<IARC, 2B; CPDB, +ve>		MN	+	Rat	iv	31–125 × 2d	[20]
			MN	–	Mouse	po	4–32	[11]
			MN	–	Mouse	iv	10–40	[11]
			UDS	–	Rat	po	75, 60 × 5d	[10,26]
			In vitro Ames/CA	+/+				[11,14]
3	*o*-Anisidine [90-04-0] (*o*-Anisidine HCl [134-29-2])		MN	–	Mouse	ip	400–800	[11]
	<IARC, 2B; CPDB, +ve>		TG (liver)	–	BigBlue mouse	po	750 × 3d	[21,31]
			UDS	–	Rat	po	50–1104	[10,30]
			In vitro Ames/CA	+/+				[11,14]
4	Azidothymidine [30516-87-1]		MN	+	Rat	po	500 × 7d	[32]
	<IARC, 2B; CPDB, +ve>		MN	+	Mouse	po	500–2000 × 3d, 200–2000 × 3d	[33]
			In vitro Ames/CA	–/+				[9]
5	Benzene [71-43-2]		MN	+	Rat	po	500–2000	[20]
	<IARC, 1; CPDB, +ve>		TG (liver)	–	BigBlue Mouse	inh	1350 ppm × 84d	[21]
			In vitro Ames/CA	–/+				[11,14]

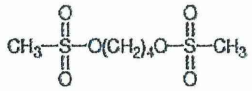
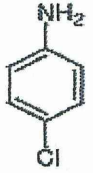
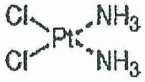
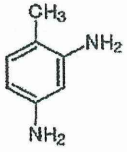
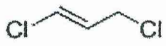
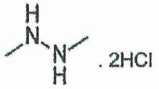
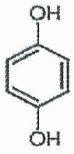
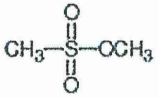
6	Busulfan (Myleran) [55-98-1]		MN	+	Mouse	ip	10–40	[11]
	<IARC, 1; CPDB, +ve>		*In vitro* Ames/CA	+/+				[11,14]
7	Cadmium chloride [10108-64-2]		MN	+	Rat	po	15, 15 × 60d	[38]
	<IARC, 1; CPDB, +ve>		*In vitro* Ames/CA	–/+				[11,14]
8	*p*-Chloroaniline [106-47-8]		MN	+	Mouse	po	300 × 3d	[40,41]
	<IARC, 2B; CPDB, +ve>		*In vitro* Ames/CA	+/+				[9,11,14]
9	Cisplatin [15663-27-1]		MN	+	Mouse	ip	0.03–10	[11]
			TG (liver)	+	LacZ mouse	ip	6	[21,43]
	<IARC, 2A; CPDB, Not listed>		*In vitro* Ames/CA	+/+				[9,11,14]
10	2,4-Diaminotoluene [95-80-7]		MN	+	Rat (PVG)	po	150–300	[45]
			MN	–	Rat (F344)	po	50–150	[45]
	<IARC, 2B; CPDB, +ve>		MN	–	Mouse	ip	30–240	[11]
			TG (liver)	+	BigBlue Mouse	po	66 × 12d	[21,46]
			UDS	+	Rat	po	150	[10,23]
			UDS	+w	Rat	po	300	[45]
			In vitro Ames/CA	+/+				[9,11,14]

Table 3 (*Continued*)

No.	Chemical [CAS] <Carcinogenicity>	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Refs.
11	1,2-Dibromoethane [106-93-4]		MN	–	Mouse	ip	25–150; 80–100 × 3d	[11]
			TG (liver)	–	MutaMouse	ip	60, 16 × 5d	[21,48]
	<IARC, 2A; CPDB, +ve>		UDS	+w	Rat	po	10–100	[10,49]
			UDS	+	Rat	ip	100	[10,49]
			In vitro Ames/CA	+/+				[11,14]
12	1,3-Dichloropropene [542-75-6]		MN	–	Rat	po	125	[52]
			MN	+	Mouse (female)	po	187, 234	[53]
	<IARC, 2B; CPDB, +ve>		UDS	–	Rat	po	125	[10,52]
			In vitro Ames/CA	+/–,+				[11,14,51]
13	1,2-Dimethylhydrazine 2HCl [306-37-6] (1,2-Dimethylhydrazine [540-73-8])		MN	+	Rat	po	200 × 2d; 25–100 × 2d	[20,56]
			UDS	+	Rat	po	20	[10,23]
	<IARC, 2A; CPDB, +ve>		*In vitro* Ames/CA	+/+				[11]
14	Hydroquinone [123-31-9]		MN	+	Mouse	po	80	[57]
	<IARC, 3; CPDB, +ve>		MN	+	Mouse	ip	30–100	[9,58]
			In vitro Ames/CA	–/+				[9,14]
15	Methyl methanesulfonate [66-27-3]		MN	+	Rat	po	36–144 × 2d	[20]
			TG (liver)	+	Mouse	ip	100	[21]
	<IARC, 2A; CPDB, +ve>		UDS	+	Rat	po	20–100	[10,23]

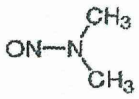
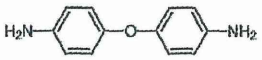
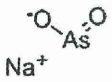
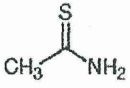
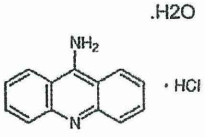
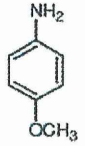
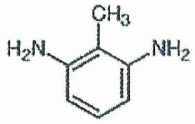
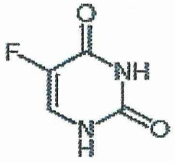
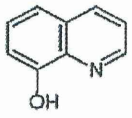
			In vitro Ames/CA	+/+			[9,11]
16	*N*-Nitrosodimethylamine [62-75-9] <IARC,2A; CPDB, +ve>		MN	+	Mouse	po	25	[61,62]
			TG (liver)	+	Mouse, Rat	po	Various doses and duration	[21]
			UDS	+	Rat	po	10	[10,23]
			In vitro Ames/CA	+/+				[9,11]
17	4,4′-Oxydianiline [101-80-4] <IARC, 2B; CPDB, +ve>		MN	+	Mouse	ip	37.5–150 × 3d	[64]
			UDS	–	Rat	po	40–725	[10,65]
			In vitro Ames/CA	+/+				[14]
18	Sodium arsenite [7784-46-5] <IARC, 1; CPDB, -ve>		MN	+	Mouse	ip	5–10	[9,66]
			In vitro Ames/CA	–/+				[9,11]
19	Thioacetamide [62-55-5] <IARC, 2B; CPDB, +ve>		MN	+	Mouse	po	50–200	[61,68]
			MN	+	Mouse	po	375–1500	[69]
			In vitro Ames/CA	–/–				[11]
	Genotoxic non-carcinogens (6)							
20	9-Aminoacridine hydrochloride monohydrate [52417-22-8] (9-Aminoacridine [90-45-9], 9-Aminoacridine HCl [134-50-9]) <IARC, Not listed; CPDB, Not listed>		No *in vivo* data					
			In vitro Ames/CA	+/+				[14]

Table 3 (*Continued*)

No.	Chemical [CAS] <Carcinogenicity>	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Refs.
21	*p*-Anisidine [104-94-9] (*p*-Anisidine HCl [20265-97-8]) <IARC, 3; CPDB, -ve>		No *in vivo* data					
			In vitro Ames/CA	+/+				[14]
22	2,6-Diaminotoluene [823-40-5] (2,6-Diaminotoluene 2HCl [15481-70-6]) <IARC, Not listed; CPDB, -ve>		MN	+w	Rat	po	300, 600	[45]
			MN	+	Mouse	ip	15.6–62.5 × 3d	[64]
			TG (liver)	–	BigBlue Mouse	diet	120 × 30d, 120 × 90d	[21,74]
			UDS	–	Rat	po	150; 150, 300	[10,23,45]
			UDS	+	Rat	po	1000, 1000 × 2d	[10,73]
			In vitro Ames/CA	+/+				[14]
23	5-Fluorouracil [51-21-8] <IARC, 3; CPDB, +ve>		MN	+	Rat	ip	20–80	[20]
			MN	+	Rat (4 wk old)	po	20, 40	[77]
			In vitro Ames/CA	–/+				[14,76]
24	8-Hydroxyquinoline [148-24-3] <IARC, 3; CPDB,-ve>		MN	–	Mouse	ip	10.8–43 × 3d	[64]
			UDS	–	Rat	po	100–500; 600, 600 × 2d	[10,73,79]
			In vitro Ames/CA	+/+				[14]

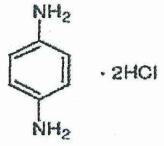
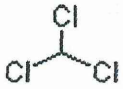
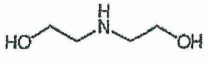
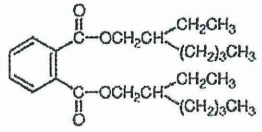
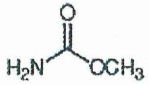
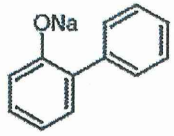
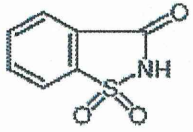
25	*p*-Phenylenediamine 2HCl [624-18-0] (*p*-Phenylenediamine [106-50-3]) <IARC, 3; CPDB, -ve>		MN	–	Rat	po	300 × 2d	[80]
			MN	–	Mouse	ip	20–100	[81]
			In vitro Ames/CA	+/+				[14]
	Non-genotoxic carcinogens (7)							
26	Chloroform [67-66-3]		MN	–	Mouse	ip	238–952 × 2d	[83]
			TG (liver)	–	BigBlue mouse	Inh	154 × 10–180d	[21]
	<IARC, 2B; CPDB, +ve>		UDS	–	Rat	po	40, 400	[10,23]
			In vitro Ames/CA	–/–				[11,14]
27	Diethanolamine [111-42-2] <IARC, 2B; CPDB, Not listed>		MN	–	Mouse	dermal	80–1250 × 90d	[9,86]
			In vitro Ames/CA	–/–				[9,14]
28	Di(2-ethylhexyl)phthalate [117-81-7]		MN	–	Mouse	ip	500–2000 × 2d	[11]
			TG (liver)	–	BigBlue mouse	diet	360–720 × 120d	[21,87]
	<IARC, 2B; CPDB, +ve>		UDS	–	Rat	po	500	[10,25]
			In vitro Ames/CA	–/–				[9,14]

Table 3 (*Continued*)

No.	Chemical [CAS] <Carcinogenicity>	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Refs.
29	Ethanol [64-17-5] <IARC, 1; CPDB, +ve>		MN	–	Mouse	drinking water	10–20% × 3–7 wk	[93,94]
			In vitro Ames/CA	–/–				[88]
30	Methyl carbamate [598-55-0] <IARC, 3; CPDB, +ve>		MN	–	Mouse	ip	500–2000, 2000–3000	[9,96]
			In vitro Ames/CA	–/–				[14]
31	*o*-Phenylphenol sodium salt [132-27-4] (*o*-Phenylphenol [90-43-7]) <IARC, 2B; CPDB, +ve; for sodium salt> <IARC, 3; CPDB, +ve; for free base>		CA	–	Rat	diet	0–2.0% × 104 wk 0–2.5% × 13 wk	[98,99]
			CA	–	Mouse	po	250–4000; 50–800 × 5d	[98]
			MN	–	Rat	diet	0–12500 ppm	[100]
			In vitro Ames/CA	–/+				[28]
32	Saccharin [81-07-2] (Saccharin sodium [128-44-9]) <IARC, 3; CPDB, +ve for sodium salt, -ve for free base>		CA	–	Mouse	po	4000	[9,103,104]
			TG (liver)	–	BigBlue rat	diet	Dose not specified (×10d)	[105]
			In vitro Ames/CA	–/–				[9]

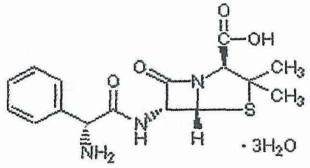
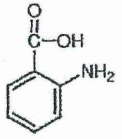
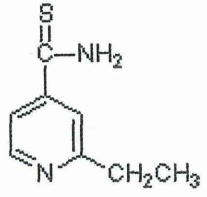
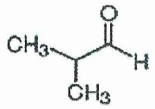
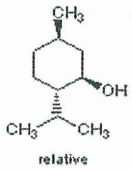
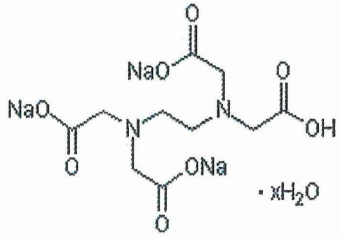
	Non-genotoxic, non-carcinogens (8)							
33	Ampicillin trihydrate [7177-48-2] (Ampicillin [69-53-4]) <IARC, 3; CPDB, -ve>		MN	–	Rat	po	3000, 5000	[9,108]
			In vitro Ames/CA	–/–				[9,14]
34	o-Anthranilic acid [118-92-3] <IARC, 3; CPDB, -ve>		MN	–	Mouse	ip	75–300, 150–600	[9,110]
			In vitro Ames/CA	–/+				[9,14]
35	*t*-Butylhydroquinone [1948-33-0] <IARC, Not listed; CPDB, -ve>		MN	–	Mouse	ip	9–400 x 3d	[9,112]
			CA	–	Mouse	ip	50–200	[112]
			In vitro Ames/CA	–/+				[9,14]
36	Ethionamide [536-33-4] <IARC, 3; CPDB, +ve; NCI, -ve>		No *in vivo* data					
			In vitro Ames/CA	–/+				[9,14]

Table 3 (*Continued*)

No.	Chemical [CAS] <Carcinogenicity>	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Refs.
37	Isobutyraldehyde [78-84-2]		MN	–	Rat	ip	313–1250 × 3d	[9,116]
			MN	–	Mouse	ip	39–1250 × 3d; 156–625 × 3d	[9,116]
	<IARC, Not listed; CPDB, -ve>		*In vitro* Ames/CA	–/+				[9,14]
38	D,L-Menthol [15356-70-4]	 relative	MN	–	Mouse	ip	250–1000 x 3d	[64]
	<IARC, Not listed; CPDB, -ve>		*In vitro* Ames/CA	–/+				[9,14]
39	Sodium chloride [7647-14-5]	NaCl	MN	–	Mouse	ip	2000	[103]
			UDS (stomach)	–	Rat	po	1000	[119]
			In vitro Ames/CA	–/–				[14]
	<IARC, Not listed; CPDB, -ve>							[121]
40	Trisodium EDTA monohydrate [10378-22-0] (EDTA [60-00-4], Trisodium EDTA trihaydarate [150-38-9], Disodium EDTA dihydrate [6381-92-6])		MN	–	Mouse	po	500–2000	[121]
			MN	–	Mouse	ip	186	[121]
			MN	+	Mouse	ip	5–20	[121]
	<IARC, Not listed; CPDB, -ve>		*In vitro* Ames/CA	–/–				[9,14]

*Genotoxic compounds are definede as chemicals which are positive in the Ames test or standered *in vivo* genotoxicity test.
Abbreviations: CA, chromosome aberration; CPDB, carcinogenic potency database; MN, erythrocyte micronucleus test.
TG, transgenic mutation test; UDS, unscheduled DNA synthesis with liver.
d, days; wk, weeks; ip, intraperitoneal; inh, inhalation; iv, intravenious; po, per os.
–, -ve, negative.
+, +ve, positive.
+w, weak positive;