Mutagenicity of carcinogenic heterocyclic amines in *Salmonella typhimurium* YG strains and transgenic rodents including *gpt* delta

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Abstract

Chemical carcinogens to humans have been usually identified by epidemiological studies on the relationships between occupational or environmental exposure to the agents and specific cancer induction. In contrast, carcinogenic heterocyclic amines were identified under the principle that mutagens in bacterial in the Ames test are possible human carcinogens. In the 1970s to 1990s, more than 10 heterocyclic amines were isolated from pyrolysates of amino acids, proteins, meat or fish as mutagens in the Ames test, and they were demonstrated as carcinogens in rodents. In the 1980s and 1990s, we have developed derivatives of the Ames tester strains that overexpressed acetyltransferase of *Salmonella typhimurium*. These strains such as *Salmonella typhimurium* YG1024 exhibited a high sensitivity to the mutagenicity of the carcinogenic heterocyclic amines. Because of the high sensitivity, YG1024 and other YG strains were used for various purposes, e.g., identification of novel heterocyclic amines, mechanisms of metabolic activation, comparison of mutagenic potencies of various heterocyclic amines, and the co-mutagenic effects. In the 1990s and 2000s, we developed transgenic mice and rats for the detection of mutagenicity of chemicals in vivo. The transgenics were generated by the introduction of reporter genes for mutations into fertilized eggs of mice and rats. We named the transgenics as *gpt* delta because the *gpt* gene of *Escherichia coli* was used for detection of point mutations such as base substitutions and frameshifts and the *red/gam* genes of λ phage were employed to detect deletion mutations. The transgenic rodents *gpt* delta and other transgenics with *lacI* or *lacZ* as reporter genes have been utilized for characterization of mutagenicity of heterocyclic amines in vivo. In this review, we summarized the in vitro mutagenicity of heterocyclic amines in *Salmonella typhimurium* YG strains and the in vivo mutagenicity in transgenic rodents. We discussed the relationships between in vitro and in vivo mutagenicity of the heterocyclic amines and their relations to the carcinogenicity.

Keywords: Heterocyclic amines, Mutagenicity, Ames test, *Salmonella typhimurium* YG strains, Acetyltransferase, Transgenic, *gpt* delta, Carcinogenicity
Background

International Agency for Research on Cancer (IARC) has listed more than 100 agents that are carcinogenic to humans (Group 1) [1]. These carcinogenic agents were identified by epidemiological studies on the relationships between specific cancer induction and occupational or environmental exposure to these agents. For example, $o$-toluidine has been recognized as a human carcinogen because of the bladder cancer of workers in dye industries [2]. Asbestos has been identified as a human carcinogen because of its strong association with mesothelioma and lung cancer in construction and factory workers [3]. 1,2-Dichloropropane was included in Group 1 agents because of bile duct cancer in employees in the printing industry [4]. Exposure to vinyl chloride monomers induces angiosarcoma in the liver of industrial workers [5]. In this regard, the discovery of carcinogenic heterocyclic amines is unique because they were initially identified as mutagens in bacteria in the Ames test and then demonstrated as carcinogens in rodents [6]. 3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), 2-amino-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1), 2-aminoimidopyrido[1,2-a:3',2'-d]imidazole (Glu-P-2), 2-amino-9H-pyrido[2,3-b]indole (AaC) and 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAaC) were isolated from the pyrolysates of amino acids and proteins as potent mutagens in Salmonella enterica subsp. enterica serovar Typhimurium (Salmonella typhimurium) TA98 (Table 1) (Supplementary Fig. 1 and Table 1) [7–9]. 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) were isolated from broiled meat or fish also as potent mutagens in strain TA98 [10–14]. Fortunately, there is no accidental excess exposure to heterocyclic amines in humans. Therefore, the link between the consumption of heterocyclic amines and human cancer is still debatable. However, IQ is ranked as a probable human carcinogen (Group 2A), and others are ranked as possible human carcinogens (Group 2B) by IARC [15]. Therefore, the history of research on carcinogenic heterocyclic amines would provide valuable lessons about

Table 1 *Salmonella typhimurium*\(^1\) YG strains

| Strain          | Description                                             | Reference |
|-----------------|---------------------------------------------------------|-----------|
| TA98            | TA1538 (pKM101)                                         | [19, 20]  |
| TA98NR          | as TA98 but deficient in classical nitroreductase       | [21]      |
| TA98/1,8-DNP \(_6\) | as TA98 but deficient in O-acetyltransferase              | [21]      |
| TA1538/NR       | as TA1538 but deficient in classical nitroreductase     | [22]      |
| TA1538/1,8-DNP | as TA1538 but deficient in O-acetyltransferase           | [22]      |
| YG1006          | TA1538/1,8-DNP (pYG121)\(^2\)                          | [16]      |
| YG1012          | TA1538/1,8-DNP (pYG213)\(^3\)                          | [23]      |
| YG1019          | TA1538/1,8-DNP (pYG219)\(^4\)                          | [23]      |
| YG1020          | TA98 (pBR322-AP)                                        | [16]      |
| YG1021          | TA98 (pYG216)\(^5\): a nitroreductase-overproducing strain | [24]      |
| YG1024          | TA98 (pYG219)\(^6\): an O-acetyltransferase-overproducing strain | [16]      |
| YG1024NR        | TA98NR (pYG121): TA98NR (nitroreductase-deficient) but O-acetyltransferase is overexpressed | [25]      |
| YG1041          | TA98 (pYG233): a nitroreductase and O-acetyltransferase-overproducing strain | [26]      |
| TA100           | TA1535 (pKM101)                                         | [19, 20]  |
| TA100NR         | as TA100 but deficient in classical nitroreductase      | [21]      |
| TA100/1,8-DNP   | as TA100 but deficient in O-acetyltransferase           | [21]      |
| YG1025          | TA100 (pBR322-AP)                                        | [16]      |
| YG1026          | TA100 (pYG216): a nitroreductase-overproducing strain   | [24]      |
| YG1029          | TA100 (pYG219): an O-acetyltransferase-overproducing strain | [16]      |
| YG1042          | TA100 (pYG233): a nitroreductase and O-acetyltransferase-overproducing strain | [26]      |

\(^1\) The formal name *Salmonella enterica* subsp. enterica serovar Typhimurium is abbreviated as *Salmonella typhimurium* in Tables, Figures and the text

\(^2\) Plasmid pYG121 is a derivative of plasmid pBR322 having part of the chromosome of TA1538 including the *oat* gene encoding O-acetyltransferase

\(^3\) Plasmid pYG213 is a deletion derivative of pYG122. Plasmid pYG121, 122, 213 have the ampicillin-resistance gene

\(^4\) Plasmid pYG219 is a derivative of pBR322 having 1.35 kb DNA containing the *oat* gene from pYG213. It has the tetracycline-resistance gene

\(^5\) Plasmid pYG216 is a derivative of pBR322 having 6.85 kb DNA containing the *cnr* gene encoding the classical nitroreductase in strain TA1538. Plasmid pYG216 has the tetracycline-resistance gene

\(^6\) Plasmid pYG233 is a derivative of pBR322 having the *oat* and *cnr* genes. Plasmid pYG233 has the tetracycline-resistance gene
the roles of in vitro and in vivo mutagenicity assays in the discovery of human carcinogens. In this review, we have first summarized in vitro mutagenicity of heterocyclic amines in Salmonella typhimurium YG strains that are highly sensitive to the mutagenicity of aromatic amines and nitro aromatics [16]. Second, we have presented the review of in vivo mutagenicity of heterocyclic amines in transgenic rodents such as gpt delta mice/rats, lacI mice/rats and lacZ mice [17, 18]. Finally, we have discussed the effectiveness and limitations of the mutagenicity assays to discover human carcinogens and the cancer risk of heterocyclic amines in daily life.

Review
Development of Salmonella typhimurium YG strains

In the 1970s, Dr. Bruce N. Ames, University of California, developed a bacterial mutagenicity test (Ames test) and reported that a high percentage of bacterial mutagens in the Ames test are rodent carcinogens [27, 28]. The test is simple, rapid and economical; therefore, large number of environmental chemicals were tested for potential mutagenicity in the Ames tester strains. Typical tester strains of the Ames test are Salmonella typhimurium TA98 and TA100, which detect frameshift-type mutagens and base-substitution-type mutagens, respectively [19, 20]. In the same era, Dr. Takashi Sugimura, National Cancer Center in Japan, was interested in the possibility that smoke from broiled fish might be mutagenic and carcinogenic. Dr. Sugimura and his collaborators examined this possibility using the Ames test and isolated many heterocyclic amines as mutagens from pyrolysates of amino acids, proteins, meat or fish as mutagens [6, 29]. Similarly, Dr. Daisuke Yoshida, the Japan Tobacco & Salt Public Cooperation, isolated AaC and MeAaC from pyrolys products of soybean globulin and Dr. James S. Felton, Lawrence Livermore National Laboratory, U.S.A., identified PhIP and the related chemicals from fried ground beef [9, 14].

Heterocyclic amines require metabolic activation for mutagenesis and carcinogenesis. In general, they are first oxidized by CYP1A2 to N-hydroxy derivatives, which are further activated by O-acetyltransferase or sulfotransferase to the nitrenium ions, thereby inducing DNA adducts and mutations [30–33]. In the Ames test, these metabolic enzymes are provided as 9,000 x g supernatant of rat liver homogenates (S9) [34]. It must be pointed out, however, Salmonella typhimurium used in the Ames test has enzymes involved in metabolic activation. In fact, strain TA98/1,8-DNP₆ is significantly resistant to the mutagenicity and killing effects of aromatic amines and nitro aromatics, because this strain is devoid of acetyltransferase activity [21].

In the mid-1980s, we were interested in the metabolic activation mechanisms of chemical carcinogens and cloned the oat gene encoding bacterial O-acetyltransferase [16, 22]. For this purpose, we constructed a gene library of Salmonella typhimurium strain TA1538 with a multicopy-number plasmid pBR322 and introduced the gene library into strain TA1538/1,8-DNP, which is the same as TA98/1,8-DNP₆ but lacks plasmid pKM101 (Fig. 1). We searched for colonies that could grow on plates without 2-nitrofluorene (2-NF) but could not grow on plates with 2-NF. The principle was that if a plasmid carrying the oat gene was introduced into the host strain TA1538/1,8-DNP, the transformants would not grow on plates with 2-NF but grow on plates without 2-NF because 2-NF requires activities of O-acetyltransferase for cytotoxicity and mutagenicity. Fortunately, we successfully isolated candidate colonies and confirmed that the sensitivity was maintained after the plasmids extracted from the candidate colonies were introduced to the fresh background of TA1538/1,8-DNP. Plasmid pYG121 and pYG122 were the first isolated plasmids that carried the oat gene (Table 1). We then constructed plasmid pYG213, a deletion derivative of pYG122, which contains a 1.35kb-DNA fragment of pYG122 including the oat gene. However, pYG213 has the ampicillin-resistance-gene and is incompatible with strains TA98 and TA100, both of which possess pKM101 that confers ampicillin resistance. Therefore, we subcloned the 1.35-kb DNA fragment into the Scal site of pBR322 and generated pYG219. Subcloning into this site disrupted the ampicillin-resistance gene and permitted the selection of pYG219 in TA98 and TA100. The resulting strains were named as YG1024 and YG1029, respectively [16]. N-hydroxy-Glu-P-1 O-acetyltransferase activities of TA1538/1,8-DNP harboring pBR322, pYG122, pYG213 or pYG219 were 0, 28.0, 228

Fig. 1 Gene cloning of the oat gene encoding O-acetyltransferase in Salmonella typhimurium. The chromosome DNA of Salmonella typhimurium TA1538 was partially digested with Sau3A1 and ligated to BamH1-digested plasmid pBR322, thereby generating a plasmid library of TA1538. Then, the library DNA was introduced into Salmonella typhimurium TA1538/1,8-DNP and screened the colonies that could grow on plates without 2-NF but could not grow on plates with 2-NF.
or 54.6 nmol/min/mg protein, respectively [16, 23]. Although strain YG1012, which is TA1538/1,8-DNP harboring pYG213, had the highest O-acetyltransferase activity, it exhibited lower sensitivity to the mutagenicity of 1-aminonaphthalene + S9, 1-nitropyrene and 1,8-dinitropyrene compared to YG1024 [23]. It suggests that these chemicals require the presence of pKM101 for maximum frameshift mutagenesis. Plasmid pKM101 carries the mutAB genes encoding DNA polymerase RI, an error-prone DNA polymerase involved in translesion DNA synthesis [35]. Owing to the possession of pKM101 and the wider range of sensitivity, strain YG1024 is more widely used for mutation assays than strain YG1012 [36]. However, YG1024 showed comparable or slightly lower sensitivity to 2-hydroxy-acetylaminofluorene, Glu-P-1 + S9 and 2-aminoanthracene +S9 compared to YG1012 [23]. It appears that these chemicals are not strongly dependent on the presence of pKM101 for maximum mutagenesis. Later, we constructed plasmid pYG233 carrying the out gene and the cnr gene encoding classical nitroreductase [24] and introduced it to strains TA98 and TA100. The resulting strains YG1041 and YG1042, respectively, overexpressed both acetyltransferase and nitroreductase [26]. They were more sensitive to the mutagenicity of nitroaromatics such as 2-NF, 2,6-dinitrotoluene and 1-nitropyrene than YG1024 or YG1029. A possible problem with YG1041 and YG1042 is the extreme sensitivity to the killing effects of nitro, amino and hydroxyamino compounds. The number of revertants increased very sharply and decreased quickly with increasing doses. In addition, the number of spontaneous revertants per plate of YG1041 and YG1042 was higher than that of spontaneous revertants per plate of YG1024 and YG1029, respectively. The high number of spontaneous revertants obscures the weak mutagenicity of chemicals. Therefore, we recommend using these strains along with other strains such as YG1024 and YG1029 to avoid overlooking the mutagenic responses of test chemicals.

**Mutagenicity of heterocyclic amines in YG strains**

**Novel heterocyclic amines**

Heterocyclic amines were initially isolated from the pyrolysates of food or food components. Later, they were isolated from various environmental sources such as river water [37], automobile exhaust particles [38], cigarette smoke [39], human excretion [40] and rainwater [41]. Appropriate devices and methods are required to efficiently collect environmental mutagens. In the case of river water, it is critical to effectively collect and concentrate the target molecules from a large volume of water samples because pollutants are present in only minute concentrations. Sakamoto and Hayatsu developed an effective method, i.e., the blue rayon hanging technique, in which blue rayon covalently bound to the blue pigment copper phthalocyanine is hung in the river to specifically adsorb polycyclic planar compounds including heterocyclic amines [42]. The blue rayon absorbing water pollutants, instead of a large volume of water samples, is transferred to laboratories for chemical analyses and mutagenicity assays. Kataoka et al. [43] isolated and identified IQ, Trp-P-1 and AaC in the Danube River in Vienna by the method. The river water samples exhibited higher mutagenicity in YG1024 than in TA98 in the presence of S9 activation, which suggested a significant contribution of the heterocyclic amines to the whole mutagenicity of the water samples (Table 2). The source of the heterocyclic amines in the Danube River may be the emission and discharge from food processing, e.g., smoke sausage, and wood burning. The collection of mutagens in river water by the blue-rayon hanging technique and the subsequent mutagenicity assays with YG1024 were conducted in samples from the Chao Phraya River in Bangkok, Thailand, and the Sumida and Ara Rivers in Tokyo [44]. Similar methods were employed for samples from rivers in Boston, New York, Washington D.C. and Montreal in North America [45]. In the latter case, YG1041 and YG1024 were much more sensitive than TA98 in the presence of S9 plus an NADPH-generating system (S9 mix).

Research on mutagens in river water led to the discovery of a novel class of heterocyclic amines. Nukaya et al. employed the blue rayon hanging technique for the collection of samples at sites below sewage plants of the Nishitakase River in Kyoto, Japan, and identified a novel mutagen, i.e., 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-1) [69]. PBTA-1 was highly mutagenic in YG1024 in the presence of S9 mix, and the mutagenic potency (revertants per μg) was equivalent to that of Glu-P-1. For the reason that there are several dye factories in Kyoto, PBTA-1 is probably produced by the treatment of wastewater from dye factories in the sewage plants. In fact, PBTA-1 can be formed from dinitropheynilazo dye used as an industrial material in textile dyeing by reduction and chlorination [70]. PBTA-1 analogs, i.e., PBTA-2, PBTA-3, PBTA-4, PBTA-5, PBTA-6, PBTA-7 and PBTA-8, were later isolated from rivers in Kyoto and Aichi, Japan [71, 73–75, 78]. All these chemicals were mutagenic in YG1024 in the presence of S9 mix and the order of mutagenic potency was PBTA-4 > PBTA-2 > PBTA-3 > PBTA-1 > PBTA-5 > PBTA-6 = PBTA-8 > PBTA-7. Despite the potent mutagenicity in the Ames test, the carcinogenicity of PBTAs in rodents has not been reported.

Another novel heterocyclic amine was isolated as a mutagen generated by the Maillard reaction of glucose and amino acids. Nishigaki et al. incubated mixtures of
| Chemical | Strain | Metabolic activation | Remarks | Reference |
|----------|--------|----------------------|---------|-----------|
| 2-amino-1-methyl-6-phenylimidazo[4,5]-pyridine (PhIP) | TA98, TA98NR, YG1024, TA98/1,8-DNP₆ | W | The mutagenic potency (revertants/μg) was in the order of YG1024 > TA98 = TA98/1,8-DNP₆ > TA98NR | [46] |
| 2-amino-1-methyl-6-phenylimidazo[4,5]-pyridine (PhIP) | TA98, YG1024 | W | The mutagenicity of PhIP was enhanced up to six times by the presence of ethylparathion, methylparathion or methyl paraxon. | [48] |
| 2-amino-1-methyl-6-phenylimidazo[4,5]-pyridine (PhIP) | YG1024 | W | PhIP was negative in both strains in the presence of colon S9 prepared from 3-MC-treated rats. | [49] |
| 2-amino-1-methyl-6-phenylimidazo[4,5]-pyridine (PhIP) | TA98, YG1024 | W* | The mutagenicity of PhIP was enhanced up to six times by the presence of ethylparathion, methylparathion or methyl paraxon. | [48] |
| 2-amino-1-methyl-6-phenylimidazo[4,5]-pyridine (PhIP) | TA1538, YG1019 | W | The mutagenic potency (revertants/μg) was in the order of MelQx = IQ > 4,8-diMeIQx > > PhIP > MeAαC > AαC in YG1019. | [51] |
| 2-amino-1-methyl-6-phenylimidazo[4,5]-pyridine (PhIP) | YG1024 | W* | The mutagenic potency of PhIP was IQ = MelQx-Trp-P-1 = MelQx>PhIP in the presence of HepG2 cell homogenates. The order was unchanged when rat S9 was used although the mutagenic potency was much more enhanced with rat S9. | [51] |
| 2-nitro-1-methyl-6-phenylimidazo[4,5]-pyridine (NO₂-PhIP) | TA98, TA98NR, YG1024, TA98/1,8-DNP₆ | W/O | The mutagenic potency (revertants/μmol) was in the order of YG1024 = TA98 > TA98/1,8-DNP₆ > TA98NR. | [46] |
| 2-azido-1-methyl-6-phenylimidazo[4,5]-pyridine (azido-PhIP) | TA98, TA98NR, YG1024, TA98/1,8-DNP₆ | W* | Near UV light was used for activation. The sensitivity (revertants/μmol) was in the order of YG1024 = TA98 = TA98NR > TA98/1,8-DNP₆. | [46] |
| 2-aminoo-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) | YG1020, YG1024, YG1025, YG1029 | W | The mutagenic potency (revertants/μmol) was in the order of YG1024 > YG1020 > YG1029 > YG1025. | [16] |
| 2-aminoo-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) | YG1024, TA98NR, TA98/1,8-DNP₆, YG1021, YG1024 | W | The mutagenic potency (revertants/μmol) was in the order of YG1024 > YG1021 > TA98 > TA98NR > TA98/1,8-DNP₆. | [36] |
| 2-aminoo-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) | YG1020, YG1024, YG1012, YG1019 | W | The mutagenic potency (revertants/μmol) was in the order of YG1012 > YG1019 = YG1024 > YG1020. | [23] |
| 2-aminoo-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) | TA98, YG1021, YG1024, YG1041 | W | The mutagenic potency (revertants/μmol) was in the order of YG1024 > YG1021 > YG1024 > YG1021. | [26] |
| 2-aminoo-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) | YG1024 | W | YG1024 may lose plasmid pYG1219 under highly toxic conditions. | [52] |
| 2-aminoo-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) | YG1006, TA98 | W* | Ram seminal vesicle microsomes (prostaglandin H synthase) activated Glu-P-1 for mutagenesis in YG1006. | [53] |
| 2-hydroxyamino-6-methylpyrido[1,2-a:3',2'-d]imidazole (N-OH-Glu-P-1) | YG1020, YG1024, YG1025, YG1029 | W/O | The mutagenic potency (revertants/μmol) was in the order of YG1024 > YG1020 = YG1029 > YG1025. S9 was not needed for the mutagenicity. | [16] |
| 2-hydroxyamino-6-methylpyrido[1,2-a:3',2'-d]imidazole (N-OH-Glu-P-1) | TA98, TA98NR, TA98/1,8-DNP₆, YG1021, YG1024 | W/O | The mutagenic potency (revertants/μmol) was in the order of YG1024 > YG1021 = TA98NR > TA98/1,8-DNP₆. S9 was not needed for the mutagenicity. | [36] |
| 2-hydroxyamino-6-methylpyrido[1,2-a:3',2'-d]imidazole (N-OH-Glu-P-1) | TA98, TA98NR, TA98/1,8-DNP₆, YG1024, YG1025 | W/O | The mutagenic potency (revertants/μmol) was in the order of YG1024 > YG1021 = TA98NR > TA98/1,8-DNP₆. S9 was not needed for the mutagenicity. | [36] |
| 2-nitro-6-methylpyrido[1,2-a:3',2'-d]imidazole (NO₂-Glu-P-1) | TA98, TA98NR, TA98/1,8-DNP₆, YG1024, YG1025 | W/O | The mutagenic potency (revertants/μmol) was in the order of YG1024 > YG1021 > TA98 > TA98/1,8-DNP₆ > TA98NR. | [36] |
| 2-nitro-6-methylpyrido[1,2-a:3',2'-d]imidazole (NO₂-Glu-P-1) | TA98, TA98NR, TA98/1,8-DNP₆, YG1024, YG1025 | W/O | The mutagenic potency (revertants/μmol) was in the order of YG1024 > YG1021 > TA98 > TA98/1,8-DNP₆ > TA98NR. | [36] |
| 3-amino-1,4-dimethyl-SR-pyrido[4,3-b]indole (Trp-P-1) | YG1024 | W* | The mutagenic potency was IQ = MelQx-Trp-P-1 = MelQx>PhIP in the presence of HepG2 cell homogenates. The order was unchanged when rat S9 was used although the mutagenic potency was much more enhanced with rat S9. | [51] |
| Chemical                                                                 | Strain          | Metabolic activation | Remarks                                                                                       | Reference     |
|-------------------------------------------------------------------------|-----------------|----------------------|----------------------------------------------------------------------------------------------|---------------|
| 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1)                    | TA98, YG1024    | W                    | Trp-P-1 was identified in samples from the Danube River in Vienna.                           | [43]          |
| 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2)                       | TA98, TA98NR,   | W                    | The sensitivity (induced revertants/nmol) was in the order of TA98/1,8-DNP₆ > YG1021 = TA98 > YG1024 = TA98NR. | [36]          |
|                                                                         | YG1021, YG1024  |                      |                                                                                              |               |
| 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2)                       | TA98, YG1021,   | W                    | The sensitivity (induced revertants/nmol) was not substantially different among the four strains. | [26]          |
|                                                                         | YG1024, YG1041  |                      |                                                                                              |               |
| 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2)                       | TA98, YG1024    | W                    | Untreated rat liver S12 fraction was used for metabolic activation. The sensitivity was similar between TA98 and YG1024. | [54]          |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                             | YG1012          | W*                   | Human or rat cytochrome P-450 1A2 plus hydrogen peroxide supported metabolic activation of IQ. | [55]          |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                             | TA1538, YG1019  | W                    | The mutagenic potency (revertants/ng) was in the order of MeIQx = IQ > 4,8-diMeIQx>> > PhIP>MeAαC>AαC in YG1019. | [50]          |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                             | TA98, YG1024,   | W                    | The sensitivity (induced revertants/nmol) was in the order of YG1024> > TA98> > TA98/1,8-DNP₆.   | [46]          |
|                                                                         | TA98/1,8-DNP₆   |                      |                                                                                              |               |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                             | YG1024          | W*                   | Ram seminal vesicle microsomes(supplemented with arachidonic acid) activated IQ for mutagenesis. | [56]          |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                             | YG1024, TA98    | W                    | The sensitivity (induced revertants/nmol) was in the order of YG1024 > TA98.                  | [57]          |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                             | YG1024          | W*                   | The mutagenic potency was IQ = MeIQ>Trp-P-1 = MeIQx>PhIP in the presence of HepG2 cell homogenates. The order was unchanged when rat S9 was used although the mutagenic potency was much more enhanced with rat S9. | [51]          |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                             | YG1024          | W*                   | The C8-dG-IQ-adduct N-(deoxyguanosin-8-yl)-IQ was the major adduct when IQ was incubated with YG1024 either in ovine seminal vesicle cells (prostaglandin H synthase) or hepatocytes (monooxygenases). | [58]          |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                             | YG1024          | W                    | Urinary metabolites of IQ-treated rats were investigated with improved extraction methods and assay conditions. | [59]          |
| 2-nitro-3-methylimidazo[4,5-f]quinoline (NO₂-IQ)                         | TA98, YG1024,   | W/O                  | The sensitivity (induced revertants/nmol) was in the order of YG1024> > TA98> > TA98/1,8-DNP₆.   | [46]          |
|                                                                         | TA98/1,8-DNP₆   |                      |                                                                                              |               |
| 2-nitro-3-methylimidazo[4,5-f]quinoline (NO₂-IQ)                         | YG1024          | W/O                  | NO₂-IQ and NO-IQ exhibited similar mutagenicity to YG1024.                                   | [56]          |
| 2-nitroso-3-methylimidazo[4,5-f]quinoline (NO-IQ)                        | YG1012, YG1024, | W/O                  | NO₂-IQ was a metabolite of IQ by ram seminal vesicle microsomes (prostaglandin H synthase). YG1012 exhibited similar or slightly higher sensitivity to NO₂-IQ than YG1024. | [25]          |
|                                                                         | YG1024NR        |                      |                                                                                              |               |
| 2-nitroso-3-methylimidazo[4,5-f]quinoline (NO-IQ)                        | YG1024          | W/O                  | NO₂-IQ and NO-IQ showed similar mutagenicity to YG1024.                                       | [56]          |
| 7-hydroxy-2-amino-3-methylimidazo[4,5-f]quinoline (7-OH-IQ)              | YG1012, YG1024NR| W/O                 | 7-OH-IQ was a possible metabolite of IQ by ram seminal vesicle microsomes. The mutagenicity was substantially lower than that of NO₂-IQ. | [25]          |
Table 2 Mutagenicity of heterocyclic amines in *Salmonella typhimurium* YG strains (Continued)

| Chemical | Strain | Metabolic activation | Remarks | Reference |
|----------|--------|----------------------|---------|-----------|
| 2, 2′-azo-bis-3-methylimidazo[4,5-f]quinoline (azo-IQ) | YG1024, TA98 | W/O | azo-IQ was a metabolite of IQ in the presence of ram seminal vesicle microsomes. The mutagenicity was much weaker than NO-IQ or NO2-IQ. | [56] |
| 2-amino-3-methylimidazo[4,5-f]quinoxaline (IQx) | YG1024, TA98 | W | The mutagenic potency of IQx (revertants/μg) was more than 50 times higher than that of 1-methynaphtho[2,3-d]imidazole-2-amine (Linear-NI). | [50] |
| 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) | YG1024, TA98 | W | YG1024 may lose plasmid pYG219 under highly toxic conditions. | [52] |
| 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) | YG1024, TA98 | W* | The sensitivity (induced revertants/nmol) was in the order of YG1024 > TA98. | [57] |
| 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) | YG1024, TA98 | W* | Untreated rat liver S12 fraction was used for metabolic activation. The sensitivity was similar between TA98 and YG1024 because of the high toxicity. | [54] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024, TA98 | W* | The mutagenic potency was IQ = MeIQ>Trp-P-1 = MeIQx>PhIP in the presence of HepG2 cell homogenates. The order was unchanged when rat S9 was used although the mutagenic potency was much more enhanced with rat S9. | [51] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024, TA98 | W* | The mutagenic potency was IQ = MeIQ>Trp-P-1 = MeIQx>PhIP in the presence of HepG2 cell homogenates. The order was unchanged when rat S9 was used although the mutagenic potency was much more enhanced with rat S9. | [50] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1006, TA98 | W* | Ram seminal vesicle microsomes (prostaglandin H synthase) activated MeIQ for mutagenesis in YG1006. | [53] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | TA1538, YG1019 | W | The mutagenic potency (revertants/ng) was in the order of MeIQx = IQ > 4,8-diMeIQx >> > PhIP > MeAβC > AcAeC in YG1019. | [50] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024, TA98 | W* | MeIQx was mutagenic to YG1024 in the presence of human liver microsomes. YG1024 was more sensitive than TA98. N-OH-MeIQx was a major oxidation product by human liver microsomes. | [62] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024, TA98 | W | The sensitivity (induced revertants/μg) was about 12 times higher in YG1024 than in TA98. | [57] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024 | W* | The mutagenic potency was IQ = MeIQ>Trp-P-1 = MeIQx>PhIP in the presence of HepG2 cell homogenates. The order was unchanged when rat S9 was used although the mutagenic potency was much more enhanced with rat S9. | [51] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024 | W | The sensitivity (induced revertants/nmol) was in the order of YG1024 > TA98. | [57] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024 | W | The sensitivity was about 16 times higher in YG1024 than in TA98. The mutagenic potency (induced revertants/μg) was more than 4000 times lower than that of MeIQx. | [47] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024, TA98 | W | The mutagenic potency (revertants/ng) was in the order of MeIQx = IQ > 4,8-diMeIQx >> > PhIP > MeAβC > AcAeC in YG1019. | [50] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024 | W | The mutagenic potency (induced revertants/μg) was about 12 times higher in YG1024 than in TA98. | [47] |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | YG1024 | W | The sensitivity was about 16 times higher in YG1024 than in TA98. The mutagenic potency (induced revertants/μg) was more than 4000 times lower than that of MeIQx. | [47] |
| Chemical                                                      | Strain         | Metabolic activation | Remarks                                                                                   | Reference |
|---------------------------------------------------------------|----------------|----------------------|-------------------------------------------------------------------------------------------|-----------|
| 2-amino-1,7,9-trimethylimidazo[4,5-g]quinoxaline (7,9-diMeIQx) | YG1024, TA98   | W                    | The sensitivity was about 4 times higher in YG1024 than in TA98. The mutagenic potency (induced revertants/μg) was more than 100 times lower than that of MeIQx. | [47]      |
| 9-(4′-aminophenyl)-9H-pyrido[3,4-b]indole (aminophenylnorharman, APNH) | TA98, YG1024, TA100, YG1029 | W                    | APNH was formed from aniline and norharman in the presence of S9 mix. The sensitivity was YG1024 > TA98 > YG1029 > TA100. The mutagenic potency of APNH (revertants/μg) was comparable to those of MeIQx and Glu-P-1. | [65]      |
| 9-(4′-hydroxyaminophenyl)-9H-pyrido[3,4-b]indole (hydroxynamino-phenylnorharman) | YG1024         | W/O                  | N-hydroxy derivative of APNH.                                                              | [65]      |
| 9-(4′-amino-3′-methylphenyl)-9H-pyrido[3,4-b]indole (amino-3′-methylphenylnorharman) | TA98, TA100, YG1024, YG1029 | W                    | Amino-3′-methylphenylnorharman was formed from aniline and o-toluidine in the presence of S9 mix. The sensitivity was YG1024 > TA98 > YG1029 > TA100. The mutagenic potency (revertants/μg) was weaker than that of aminophenylnorharman. | [66]      |
| 5-amino-6-hydroxy-8H-benzo[6,7]azepino[5,4,3-de]quinolin-7-one (ABAQ) | TA98, TA100, YG1024, YG1029 | W                    | ABAQ was formed by the Maillard reaction of glucose and amino acids. The sensitivity was YG1024 > TA98 > YG1029 > TA100. The mutagenic potency of ABAQ (revertants/μg) was comparable to that of PhIP. | [67]      |
| 2-amino-9H-pyrido[2,3-b]indole (AαC)                          | TA1538, YG1019 | W                    | The mutagenic potency (revertants/μg) was comparable to that of PhIP.                      | [50]      |
| 2-amino-9H-pyrido[2,3-b]indole (AαC)                          | YG1019, TA1538 | W                    | AαC is a mutagen detected in panfried or grilled meat.                                     | [68]      |
| 2-nitro-9H-pyrido[2,3-b]indole (NaC)                          | YG1019         | W/O                  | NoC was a direct-acting mutagen. The mutagenic potency in the absence of S9 was lower than that of AαC in the presence of S9. | [68]      |
| 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC)               | TA1538, YG1019 | W                    | The mutagenic potency (revertants/μg) was comparable to that of PhIP.                      | [50]      |
| 1-methylimidazo[4,5-b][1,8]naphthyridin-2-amine (compound 1)  | YG1024, TA98   | W                    | The mutagenic potency (revertants/μg) was comparable to that of PhIP.                      | [60]      |
| 1-methylimidazo[4,5-b][1,7]naphthyridin-2-amine (compound 2)  | YG1024, TA98   | W                    | Linear-NI > compound 2 > compound 5 > compound 3 > compound 4 = compound 1. The sensitivity was YG1024 > TA98. The sensitivity was YG1024 > > TA98. | [69]      |
| 1-methylimidazo[4,5-b][1,6]naphthyridin-2-amine (compound 3)  | YG1024, TA98   | W                    | Linear-NI > compound 2 > compound 5 > compound 3 = compound 4 = compound 1. The sensitivity was YG1024 > > TA98. | [69]      |
| 1-methylimidazo[4,5-g][1,5]naphthyridin-2-amine (compound 4)  | YG1024, TA98   | W                    | Linear-NI > compound 2 > compound 5 > compound 3 = compound 4 = compound 1. The sensitivity was YG1024 > > TA98. | [69]      |
| 1-methylimidazo[4,5-b]quinoline-2-amine (compound 5)          | YG1024, TA98   | W                    | Linear-NI > compound 2 > compound 5 > compound 3 = compound 4 = compound 1. The sensitivity was YG1024 > > TA98. | [69]      |
| 1-methynaphtho[2,3-d]imidazole-2-amine (linear-NI)            | YG1024, TA98   | W                    | Linear-NI > compound 2 > compound 5 > compound 3 = compound 4 = compound 1. The sensitivity was YG1024 > > TA98. | [69]      |
| 2-[2-(acetamido)-4-bis(2-methoxyethyl)aminio]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-1) | YG1024         | W                    | PBTA-1 was a novel aromatic amine mutagen isolated from river water in Kyoto. The mutagenic potency (revertants/μg) was comparable to that of Glu-P-1. | [70]      |
| 2-[2-(acetamido)-4-bis(2-methoxyethyl)aminio]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-1) | TA98, TA100, YG1024, YG1029 | W                    | The sensitivity was in the order of YG1024 > > TA98 > YG1029 > > TA100. | [70]      |
PBTA-1, PBTA-2 YG1024 W PBTA-1 and PBTA-2 were released from sewage plants in the Yodo river in Japan.

PBTA-1, PBTA-2 W PBTA-1 and PBTA-2 were released from sewage plants into the Yodo river in Japan.

PBTA-2, PBTA-3, PBTA-4, PBTA-6 YG1024, YG1029 W PBTA-3, PBTA-4 and PBTA-6 substantially contributed to the mutagenicity of river water in Kyoto. The sensitivity was YG1024 > TA98 > YG1029 > TA100.

PBTA-3, PBTA-4, PBTA-6 YG1024, YG1029 W About 5 kg PBTA-type mutagens are released per year from sewage plants in the Yodo river in Japan.

PBTA-2 was a novel aromatic amine mutagen isolated from river water in Kyoto. The sensitivity was YG1024 > TA98. The mutagen may be produced from an azo dye in dyeing factories and treatment at sewage plants.

PBTA-2 was a novel aromatic amine mutagen isolated from river water in Kyto. The sensitivity was YG1024 > TA98. The mutagen may be produced from an azo dye in dyeing factories and treatment at sewage plants.

PBTA-2, PBTA-3, PBTA-4, PBTA-6 YG1024, YG1029 W PBTA-3(revertants/μg) was about 10 times lower than that of PBTA-4.

PBTA-2, PBTA-3, PBTA-4, PBTA-6 W PBTA-3, PBTA-4 and PBTA-6 substantially contributed to the mutagenicity of river water in Fukui, Japan.

PBTA-2, PBTA-3, PBTA-4, PBTA-6 W PBTA2, PBTA-3, PBTA-4 and PBTA-6 were generated in a sawage treatment plant and released to the Uji River, Japan.

PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-6, PBTA-7, PBTA-8 W About 5 kg PBTA-type mutagens are released per year from sewage plants in the Yodo river in Japan.

PBTA-7 was detected in river water in Japan. The sensitivity was YG1024 > TA98 > YG1029 > TA100. The mutagenic potency (revertants/nmol) was comparable to that of PBTA-1.

PBTA-8 was detected in river water in Japan. The sensitivity was YG1024 > TA98 > YG1029 > TA100. The mutagenic potency (revertants/nmol) was comparable to that of PBTA-1.
glucose and tryptophan with or without the Fenton reagent and showed that the reaction produced a novel mutagen, i.e., 5-amino-6-hydroxy-8H-benzo[6,7]azepino[5,4,3-de]quinolin-7-one (ABAQ) [67]. This compound was more mutagenic in YG1024 than in TA98 in the presence of S9 mix and the mutagenic potency was comparable to that of PhIP. ABAQ was mutagenic in the liver of gpt delta mice (further detailed provided in the section of “in vivo mutagenicity of heterocyclic amines in transgenic rodents”).

**Metabolic activation of heterocyclic amines**

Heterocyclic amines require metabolic activation for mutagenesis via CYP enzymes and either O-acetyltransferase or sulfortransferase. As expected, strain YG1024 overexpressing the acetyltransferase exhibited higher sensitivity, i.e., more induced revertants per nmol or μg of chemical, than strain TA98. In fact, YG1024 showed more than 10 times higher sensitivity than TA98 for Glu-P-1, IQ, MelQ, MelQx and PBTA-1 [36, 46, 57, 62, 70]. However, YG1024 exhibited similar or only slightly higher sensitivity to PhIP and Trp-P-2 than TA98, suggesting that these heterocyclic amines are not activated by acetyltransferase [46]. Consistent with this, Wu et al. reported that CHO UV-5 cells expressing mouse CYP1A2 and human N-acetyltransferase did not exhibit any significant sensitivity or genotoxicity to PhIP [81]. Wu et al. reported in the next paper that CHO UV5 cells expressing mouse CYP1A2 and human aryl sulfortransferases, i.e., HAST1 or HAST3, exhibited higher sensitivity to the killing effects of PhIP than CHO UV5 cells expressing only mouse CYP1A2 [82]. Thus, N-hydroxy-PhIP may be activated by sulfortransferase rather than acetyltransferase.

Knasmüller et al. examined the comparative mutagenicity of several heterocyclic amines with strain YG1024 and reported that IQ and MelQ were the most potent mutagens followed by MelQx and Trp-P-1 and PhIP was the weakest mutagen [51]. This order was basically the same when strain TA98 was used [6]. Part of the reason for the weak mutagenicity of PhIP in strains YG1024 and TA98 may be its low dependency on acetyltransferase for the metabolic activation.

The crystal structure of Salmonella acetyltransferase was determined at 2.8Å resolution, and it was revealed that a Cys-His-Asp catalytic triad is involved in the catalytic mechanism [83]. The critical Cys residue is conserved between the acetyltransferase of *Salmonella typhimurium* and mammalian acetyltransferases NAT1 and NAT2 [84]. Both acetyltransferases of *Salmonella typhimurium* and mammals catalyze N-acetylation (usually inactivation) and O-acetylation (usually activation) of heterocyclic amines and the N-hydroxy derivatives [85]. Mammalian NAT1 and NAT2 are polymorphic and epidemiological studies suggest that the polymorphisms modify the risk of developing various cancers such as urinary bladder, colorectal and breast cancers.

In addition to CYP enzymes, prostaglandin-H synthase activates several heterocyclic amines. This enzyme is an arachidonic acid-dependent peroxidase and is suggested to be involved in the metabolic activation of xenobiotics in extrahepatic tissues. Ram seminal vesicle microsomes, a rich source of prostaglandin-H synthase, activate IQ and MelQ for mutagenesis [53, 56]. The mutagenicity was more sensitively detected in YG strains overexpressing Salmonella acetyltransferase, i.e., YG1006 (TA1538/1,8-DNP with pYG121) and YG1024, than in TA98. The primary mutagenic metabolite of IQ by prostaglandin-H synthase is nitro-IQ, while N-hydroxy derivatives are the active metabolites of IQ and MelQx by CYP enzymes [25, 33, 56, 62]. Since nitro-IQ and N-hydroxy IQ are further activated by acetyltransferase, the same DNA adduct, i.e., C8-dG-IQ-adduct, is formed in DNA when YG1024 is treated with prostaglandin-H synthase-oxidized IQ or hepatocyte-exposed IQ [58].

**Co-mutagenic effects**

Humans are exposed to not a single chemical but a variety of chemical agents simultaneously. In this regard, modulating effects of chemicals are important for the risk estimation of environmental mutagens. Nagao et al.  

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**Table 2 Mutagenicity of heterocyclic amines in *Salmonella typhimurium* YG strains (Continued)**

| Chemical | Strain | Metabolic activation | Remarks | Reference |
|----------|--------|----------------------|---------|-----------|
| 2-[2-(acetylamino)-4-[(2-hydroxyethy lamino)-5-methoxyphenyl]-6-amino-4-bromo-2H-benzotriazole (non-CIPBTA-3)]<sup>W</sup> | TA98, TA100, YG1024, YG1029 | W | The sensitivity was YG1024> > TA98> > YG1029 > TA100. The mutagenic potency (revertants/μg) was more than 10 times lower than that of PBTA-3. | [80] |
| 2-[2-(acetylamino)-4-diethylamin o)-5-methoxyphenyl]-6-amino-4-bromo-2H-benzotriazole (non-CIPBTA-7) | TA98, TA100, YG1024, YG1029 | W | The sensitivity was YG1024> > TA98> > YG1029 > TA100. The mutagenic potency (revertants/μg) was more than 5 times lower than that of PBTA-7. | [80] |
reported interesting observations that norharman, which is not mutagenic in the Ames test, becomes mutagenic when incubated with non-mutagenic aromatic amines such as aniline, o-toluidine or m-toluidine in the presence of S9 mix [86]. Later, it was revealed that co-incubation of norharman and aniline with S9 mix produces a novel heterocyclic amine, i.e., 9-(4'-aminophenyl)-9H-pyrido[3,4-b]indole (aminophenylnorharman, APNH), and the N-hydroxy metabolite, i.e., 9-(4'-hydroxyaminophenyl)-9H-pyrido[3,4-b]indole (hydroxyaminophenylnorharman, N-OH-APNH) [65]. APNH is mutagenic in strains TA98 and YG1024 only when S9 mix is present, while N-OH-APNH is mutagenic without S9 activation. Both chemicals yielded the same DNA adducts in the DNA of YG1024. This strain showed approximately 10 times higher sensitivity to APNH and N-OH-APNH than TA98. The mutagenic potency of APNH was comparable to those of MeIQx and Glu-P-1. Similarly, incubation of norharman and o-toluidine or m-toluidine in the presence of S9 mix generates 9-(4'-amino-3'-methylphenyl)-9H-pyrido[3,4-b]indole (amino-3'-methylphenylnorharman) and 9-(4'-amino-2'-methylphenyl)-9H-pyrido[3,4-b]indole (amino-2'-methylphenylnorharman), respectively [66]. These results suggest that non-mutagenic chemicals may become mutagenic when combined.

Development of gpt delta transgenic rodents for mutagenicity assays in vivo

In the late 1980s and the early 1990s, two transgenic mouse models were developed with E. coli lacZ or lacI as reporter genes for mutations in vivo [87, 88]. In these mouse models, i.e., Muta Mouse with lacZ and Big Blue Mouse with lacI, the λ phage DNAs with the reporter gene were integrated into the chromosome of all the cells of mice [17]. After the mice are treated with chemical agents, the phage is rescued as phage particles from the mouse genome of various organs and tissues by in vitro packaging reactions. The rescued phages are introduced into indicator E. coli strains to select mutant plaques by color selection, i.e., visual search of colorless plaques in Muta Mouse or blue color plaques in Big Blue Mouse in more than 100,000 background plaques. Transgenic mouse mutagenicity assays allow detection of mutations in any organs or tissues of mice including the liver, lung, bone marrow or testis. However, color selection is time-consuming and expensive because the visual search of plaques of different color is laborious and the chromogenic agent X-gal is expensive. To overcome this limitation, a positive selection with the cII gene of phage λ has been developed [89]. The cII gene encodes a repressor protein that controls the lysogenic and lytic cycle of λ. Mutations in the cII gene can be positively identified with an indicator E. coli strain deficient in Hfl protease. In the hfl- strain, only λ phage with inactive cII can form plaques at 24°C. In contrast, all the rescued λ phage can form plaques at 37°C regardless of the status of cII. Thus, the mutant frequency (MF) can be calculated by dividing the number of plaques formed at 24°C by the number of plaques formed at 37°C and the dilution factor. The coding size of the cII gene is approximately 300 base pairs (bps), which are approximately 1/10 of lacZ and 1/3 of lacI. Thus, DNA sequencing analysis of the mutants is feasible. The cII selection detects point mutations, i.e., base substitutions and frameshifts, but not large deletions. In addition, the cII selection is applicable to both Muta Mouse and Big Blue Mouse. Later, Big Blue Rat was developed with the same λ phage DNA, i.e., λ LIZ DNA, with the lacI and cII genes [90].

In the mid-1990s, we developed another transgenic mouse model named gpt delta by introducing λEG10 DNA into fertilized eggs of C57BL/6J mice [91]. λEG10 DNA was integrated into a single site of the mouse chromosome 17 [92, 93]. A feature of the transgenic mutation assay is the incorporation of two distinct selections for detecting different types of mutations, i.e., gpt selection for point mutations andSpi' selection for deletions (Fig. 2) [17]. The gpt selection uses the gpt gene of E. coli as a reporter gene for mutations. The gpt gene is a bacterial counterpart of the human Hprt gene and encodes guanine phosphoribosyltransferase. When the gpt gene is inactivated by mutations, E. coli cells can survive on the plates containing 6-thioguanine (6-TG), whereas E. coli cells with the wild-type gpt gene cannot survive on the plates because they phosphoribosylate 6-TG to a toxic substance, i.e., 6-TGMP. Thus, the gpt selection is a positive selection. The coding size of the gpt gene is 456 bp, which is convenient for DNA sequencing analysis. The Spi' selection positively detects deletion mutations in λ phage [94]. The selection name Spi' stands for “sensitive to P2 interference”. This selection takes advantage of the restricted growth of the wild-type λ phage in P2 lysogens, which is E. coli cells carrying prophage P2 in the chromosome. Only mutant λ deficient in the functions of both the gam gene and the redBA genes can grow well in P2 lysogens and display the Spi' phenotype. Because the gam gene and the redBA genes are located side by side in the λ genome, inactivation of both functions is most likely to be induced by deletions in the region. Because of the size limitation of the λ phage in in vitro packaging reactions, the size of deletions detectable by the selection is less than 10 kb. However, tandem array of multiple copies of 48-kb λEG10 DNA in the chromosome amounts to a potential target of more than 1 mega bps. Deletion mutations with a molecular size of more than 1 kb were detected by the Spi' selection in various organs such as the liver, spleen, kidney or brain.
of the mice irradiated with heavy-ions, gamma-rays or X-rays [95–97]. Ultraviolet-B irradiation and treatment with mitomycin C also induced large deletions in the epidermis and bone marrow, respectively [98, 99]. The molecular nature of the deletion mutations can be characterized by DNA sequencing of the mutated gam and redBA region [100]. Some of the Spi− large deletions have junctions of two broken ends overlapping with short homologous sequences, while others have flush ends. It suggests that non-homologous end-joining plays an essential role in the induction of deletion mutations. The Spi− selection also detects -1 frameshifts in the gam gene that interfere with the start of translation of the downstream redBA genes [95]. The -1 frameshifts mostly occur in run sequences such as AAAAAA to AAAAA in the gam gene, and this type of mutation accounts for most of the spontaneous Spi− mutations.

In the early 2000s, Hayashi et al. introduced λEG10 DNA into fertilized eggs of Sprague-Dawley (SD) rats and established SD gpt delta rats [101]. λEG10 DNA was integrated into a single site of the chromosome 4 [93]. The SD gpt delta rats were crossed with Fischer 344 (F344) rats for 15 generations and established F344 gpt delta rats [102]. Unlike gpt delta mice, which have λEG10 DNA in both alleles of chromosome 17, gpt delta rats are heterozygous, where λEG10 is integrated into only one allele of chromosome 4. This is because homozygous rats are defective in tooth development and cannot survive after weaning. To overcome this limitation, a new homozygous gpt delta rat strain was established in the genetic background of Wistar Hannover [103]. In the new version of gpt delta rat, λEG10 was integrated into both alleles of chromosome 1 and exhibited a significantly higher packaging efficiency than the heterozygous gpt delta rats. The average of spontaneous gpt and Spi− MFs in the liver of heterozygous and new homozygous gpt delta rats are 4.4-6.5 x 10⁻⁶ and 2.8-5.5 x 10⁻⁶, respectively, which are significantly lower than those of the lacI and cII genes [104]. The low frequencies of spontaneous MFs of gpt and Spi− are similar to those of gpt delta mice. Transgenic mouse and rat mutation assays with gpt delta mouse/rat, Big Blue mouse/rat and Muta Mouse are recommended for regulatory genotoxicity assays in vivo in OECD Test Guideline 488 [105]. For the reason that rats are more frequently used for toxicological studies and cancer bioassays than mice, the transgenic rat mutation assays are expected to be combined with 28-day repeated-dose toxicity studies [106].

In vivo mutagenicity of heterocyclic amines in transgenic rodents

Organ specificity and gender difference

PhIP is the most abundant mutagenic and carcinogenic heterocyclic amine produced in cooked meat and fish [14]. It induces colon and prostate cancers in male F344 rats and mammary cancer, but not colon cancer, in female rats [107, 108]. Okonogi et al. [109] examined the mutagenicity of PhIP in the colon of male and female Big Blue rats and concluded that the MFs in the colon mucosa were enhanced by treatment with PhIP, but there were no gender differences in the MFs (Table 3). Masumura et al. [110] examined the organ specificity of
Table 3 In vivo mutagenicity of heterocyclic amines in transgenic mice and rats

| Chemical | Species | Gender | Administration | Selection | Organ | Dose | Judgement | Remarks |
|----------|---------|--------|----------------|-----------|-------|------|-----------|---------|
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | Muta Mouse, i.e., (BALB/c x DBA/2)CD2 F1 mice, but was heterozygous at the endogenous Dlb-1 locus. | male and female | Diet for 30, 60 and 90 days and sacrificed between 1 and 3 weeks after the last treatments. Diet for 30 days at a dose of 250 ppm. | lacI | small intestine | 100 ppm | + | Accumulation of mutations at both loci (lacI and Dlb-1) appears to be linear with both PhIP concentration and duration of exposure. PhIP was more mutagenic in the small intestine than in the colon. |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | Muta Mouse = (BALB/c x DBA/2)CD2 F1 mice, but was heterozygous at the endogenous Dlb-1 locus. | male and female | Gavage for 4 days, and sacrificed 7 days later. | lacZ | large intestine | 20 mg/kg/day | + | No mutagenicity was observed in any organs examined at doses of 2 and 0.2 mg/kg/day. |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | F344 rats | male and female | diet for 60 days | lacI | colon | 400 ppm | + | About 2/3 (65%) of the induced mutations were base substitutions and about half were G:C to T:A transversions. DNA was obtained from Lynch et al. [113]. |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | (C57BL/6 x SWRF1) F1 mice | male and female | diet for 60 days | lacI | intestine | 20 mg/kg/day | + | GC deletions including deletions at 5′-GGGA-3′ were the most frequent mutations. No significant difference was observed in MF between male and female. |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | C57BL6/J mice | male and female | diet for 13 weeks | got and Spi− | colon | 400 ppm | + | GC to TA transversions in runs of guanine were the hot spot of base substitutions. DNA was obtained from Zhang et al. [112]. |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | (BBR x SD) F1 rats | female | gavage for 2 weeks (5 consecutive days per week) | lacI | Mammary duct | 65 mg/kg/day | + | The highest MF was observed in the colon followed by the spleen and the liver in both got and Spi− selections. There were no gender differences in MF in the colon and the liver. |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | C57 BL6/1 mice | male | diet for 13 weeks | got and Spi− | colon | 400 ppm | + | GC to TA transversions were the most frequent mutations, followed by GC deletions including deletions at GGGA sequence. |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | F344 rats | male and female | diet for 60 days | cII | colon | 400 ppm | + | GC to TA transversions and single GC deletions were the most frequently observed mutations by got and Spi− selections, respectively. DNA was obtained from Masumura et al. [110]. |
| 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) | F344 rats | male | diet for 61 days | lacI | prostate | 200 ppm | + | Mutation spectra were similar between cII and lacI assays. GC to TA transversions were the most frequent mutations followed by GC to CG transversions, GC to AT transitions and GC deletions. |

References:
[112] Nohmi and Watanabe. Genes and Environment (2021) 43:38 Page 13 of 24
| Chemical Species                                                      | Gender       | Administration                | Selection Organ | Dose (ppm) | Judgement | Remarks                                                                 | Reference |
|---------------------------------------------------------------------|--------------|-------------------------------|-----------------|------------|-----------|--------------------------------------------------------------------------|-----------|
| 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)             | male and female | diet for 61 days              | cecum           | 200        | +                     | MF in the colon was higher than that in the cecum. G:C to T:A transversions were the most frequent mutations, followed by G:C to C:G transversions and G:C deletions. No differences in MF were observed between male and female in the colon although tumors were induced in the colon of male rats. Hormone may play a role in the induction of tumors in the colon of male rats. | [111]     |
|                                                                    |              |                               | proximal colon  | 200        | +                     |                                           |           |
|                                                                    |              |                               | distal colon    | 200        | +                     |                                           |           |
| 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)             | male and female | diet for 47 days              | distal colon    | 100        | +                     | The rats were fed a diet with conjugated linoleic acid (CLA, 0.5%, wt/wt) or 1,2-dithiole-3-thione (DTT, 0.005%, wt/wt) one week before PhIP treatments of 61 days. CLA and DTT significantly reduced PhIP-induced MF in the distal colon. In contrast, DTT significantly elevated MF in the cecum. | [120]     |
|                                                                    |              |                               | cecum           | 100        | +                     |                                           |           |
| 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)             | male and female | diet for 47 days              | kidney          | 100        | +                     | MF in male was significantly higher than that in female. Conjugated linoleic acid inhibited the mutation in female but not in male. | [121]     |
| 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)             | female       | oral dosing for 12 days       | mammary gland   | 75         | mg/kg +               | Young (43-day-old) and aged (150-day-old) female rats exhibited similar PhIP-induced MFs in the mammary gland. | [122]     |
| 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)             | male and female | intraperitoneal injection     | liver           | 70         | mg/kg –               | Calory restriction with a 40%-reduced diet for 22 weeks did not affect PhIP-induced MF in the colon. | [124]     |
|                                                                    |              |                               | kidney          | –          | +                     | MFs were significantly elevated by PhIP treatments in the colon, the spleen, the seminal vesicles and all lobes of the prostate. The MFs were higher in 8-week treatments than in 4-week treatments. |           |
| 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)             | male         | gavage for three times a week for 4 weeks or 8 weeks | liver           | 20         | mg/kg/ day +          | c-myc appears to enhance IQ-induced MF in the liver. | [126]     |
|                                                                    |              |                               | kidney          | –          | –                     |                                           |           |
| 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)             | male         | p.o. for 10 days and sacrificed 4 weeks after the dosing | liver           | 20         | mg/kg/ day +          | MF was highest in the liver, followed by the colon and the kidney. GC transversions in the liver and the colon | [127]     |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                        | male and female | gavage for a single day or for 5 consecutive days | liver           | 20         | mg/kg/ day +          |                                           |           |
|                                                                    |              |                               | kidney          | –          | +                     |                                           |           |
|                                                                    |              |                               | seminal vesicle | –          | +                     |                                           |           |
|                                                                    |              |                               | colon           | –          | +                     |                                           |           |
|                                                                    |              |                               | spleen          | +          | +                     |                                           |           |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)                        | male         | gavage for a single day or for 5 consecutive days | liver           | 20         | mg/kg/ day +          |                                           | [125]     |
|                                                                    |              |                               | kidney          | –          | +                     |                                           |           |
|                                                                    |              |                               | seminal vesicle | –          | +                     |                                           |           |
|                                                                    |              |                               | colon           | –          | +                     |                                           |           |
|                                                                    |              |                               | spleen          | +          | +                     |                                           |           |
| C57Bl/6lacZ/ (= C57Bl x Muta Mouse) and c-myc/lacZ/ (= C57Bl/6/j x CBA/J x Muta Mouse)  | ?            | p.o. for 10 days and sacrificed 4 weeks after the dosing | lacZ            | 20         | mg/kg/ day +          | c-myc appears to enhance IQ-induced MF in the liver. | [126]     |
### Table 3 In vivo mutagenicity of heterocyclic amines in transgenic mice and rats (Continued)

| Chemical | Species | Gender | Administration | Selection | Organ            | Dose             | Judgement | Remarks                                                                 | Reference |
|----------|---------|--------|----------------|-----------|------------------|------------------|-----------|--------------------------------------------------------------------------|-----------|
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) | F344 rats | male | diet for 3 weeks | cII       | liver            | 20 mg/kg/day    | +         | IQ induced higher MF in the liver than in the colon.                      | [128]     |
|         |         |        |                 |           | colon            | 20 mg/kg/day     | –         |                                                                          |           |
|         |         |        |                 |           | colon            | 70 mg/kg/day     | +         |                                                                          |           |
|         |         |        |                 |           | colon            | 200 mg/kg/day    | +         |                                                                          |           |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) | F344 rats | male | diet for 3 weeks | lacI      | colon            | 70 ppm          | +         |                                                                          | [129]     |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) | S.D. rats | female | diet for 13 weeks | gpt       | liver            | 300 ppm          | +         | GC to TA transversions and single guanine deletions were induced by IQ.   | [130]     |
| 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) | F344 rats | male | diet for 4 weeks | gpt and Spi<sup>−</sup> | liver | 0.1 ppm         | –         | Spi<sup>−</sup> selection and GST-P positive foci assay were positive only in 100 ppm, while gpt assay was positive in 10 and 100 ppm. Frequencies of GC to TA transversions in the gpt gene were significantly increased in 1, 10 and 100 ppm in a dose-dependent manner. | [131]     |
|         |         |        |                 |           | bone marrow      | 1 ppm            | –         |                                                                          |           |
|         |         |        |                 |           | bone marrow      | 10 ppm           | +         |                                                                          |           |
|         |         |        |                 |           | bone marrow      | 100 ppm          | +         |                                                                          |           |
| 2-Amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) | C57BL/6 mice | female | diet for 12 weeks | lacI      | liver            | 300 ppm          | +         | GC to TA transversions, followed by GC to AT transitions, were induced in the liver and the bone marrow. DNA was obtained from Suzuki et al. [132]. | [133]     |
|         |         |        |                 |           | bone marrow      | 300 ppm          | +         |                                                                          |           |
| 2-Amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) | C57BL/6 mice | female | diet for 1, 4, 12 weeks | lacI | liver | 300 ppm | + | MF was in the order of the colon, the bone marrow, the liver and the stomach. The MF increased in a feeding period-dependent manner. No mutagenicity was observed in the heart. | [132]     |
|         |         |        |                 |           | bone marrow  | 300 ppm | + |                                                                          |           |
|         |         |        |                 |           | bone marrow  | 300 ppm | + |                                                                          |           |
|         |         |        |                 |           | fore stomatch | 300 ppm | + |                                                                          |           |
|         |         |        |                 |           | colon      | 300 ppm | + |                                                                          |           |
|         |         |        |                 |           | colon      | 300 ppm | – |                                                                          |           |
| 2-Amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) | C57BL/6 mice | female | diet for 84 days | lacI      | colon            | 300 ppm          | +         | GC to TA transversions at 5'<sup>−</sup>GC-3'<sup>−</sup> were the hot spot of base substitutions. DNA was obtained from Suzuki et al. [132]. | [135]     |
| 2-Amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx) | C57Bl/6 lacZ (= C57Bl x Muta Mouse) and c-myc/lacZ (= C57Bl/6 x CBA/J x Muta Mouse) | ? | p.o. for 10 days, and sacrificed 4 weeks after the dosing | lacZ | liver | 20 mg/kg/day | + | c-myc appears to enhance MeIQx-induced MF in the liver. | [126]     |
| Chemical Species | Gender Administration | Selection Organ | Dose | Judgement | Remarks |
|------------------|------------------------|-----------------|------|-----------|---------|
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | male and female diet for 30 or 40 weeks | lacZ liver | 600 ppm | + | MFs in the mice fed diets containing MeIQx for 30 or 40 weeks were about 40 times higher than those of untreated mice. MF in male was higher than that in female. There was a synergistic effects of MeIQx and c-myc for hepatocarcinogenesis. c-myc also enhanced MeIQx-induced MF. |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | male single intragastric administration | lacI liver | 100 mg/kg – 100 mg/kg – | – | No mutagenicity was observed by single intragastric administration. Mutagenicity was observed only in female by 4 weeks administration. Both sexes were positive in 12 weeks administration. MF was higher in female than in male mice, which is consistent with the sensitivity to carcinogenicity of MeIQx. |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | male and female diet for 12 weeks | lacI liver | 3 ppm – 30 ppm + female/– male | – | The Liver was more sensitive to the mutagenicity of MeIQx than the colon. MF at 300 ppm for 78 weeks in liver was higher than that at the same dose for 12 weeks. Whole gpt MF and the frequencies of GC to TA were not significantly increased at 3 ppm. |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | female | diet for 12 weeks | gpt liver | 10 ppm + G:C to T:A transversions | MF was increased at 10 and 100 ppm. GST-P positive foci were induced at 100 ppm only. Most frequently induced mutations were frameshifts in guanine bases, followed by G to T transversions. |
| 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) | female diet for 13 weeks with or without CCl4 (1 ml/kg once a week) | gpt liver | 300 ppm | + | GC to TA transversions were induced by MeIQx. CCl4 treatments enhanced MF even in the p53-deficient background. |
| 9-(4'-aminophenyl)-9H-pyrido[3,4-b]indole (aminophenylnorharman, APNH) | male | diet for 12 weeks | gpt liver | 10 ppm + | GC to TA transversions, followed by GC to AT transitions, were the most frequent mutations detected by gpt assay. Single G deletions in run sequences were detected by Spi− selection. The liver was more sensitive than the colon in terms of induction of mutations. |
| 5-amino-6-hydroxy-8H-benzo[6,7]azepino[5,4,3-de]quinoxalin-7-one (ABAQ) | male | gavage for 3 weeks (5 consecutive days per week) | gpt liver | 25 mg/kg | GC to AT transitions and AT to CG transversions were increased in the liver. Both gpt and Spi− assays were positive in the liver but negative in the kidney. |
Table 3 In vivo mutagenicity of heterocyclic amines in transgenic mice and rats (Continued)

| Chemical                | Species                        | Gender     | Administration | Selection | Organ          | Dose      | Judgement | Remarks               | Reference |
|-------------------------|--------------------------------|------------|----------------|-----------|----------------|-----------|-----------|-----------------------|-----------|
| 2-amino-9H-pyrido(2,3-b)indole (AaC) | C57Bl/6/aac2 F1 (C57Bl/6 x Muta Mouse) | male and female | diet for 30 and 45 days | lacI       | liver          | 800 ppm   | +         | GC to TA transversions at 5’-CGT-3’ was the hot spot of base substitutions. DNA was obtained from Zhang et al. [112] | [112]     |
| 2-amino-9H-pyrido(2,3-b)indole (AaC) | Muta Mouse, i.e., BALB/c x DBA/2ICD2 F1 mice, but was heterozygous at the endogenous Dlb-1 locus. | male and female | diet for 30 and 45 days | lacI       | small intestine | 800 ppm   | –         | AaC was mutagenic in the colon but not in the small intestine. | [112]     |
| 2-amino-9H-pyrido(2,3-b)indole (AaC) | (C57BL/6 x SWR) F1 mice | male and female | diet for 45 days | lacI       | colon          | 800 ppm   | +         |                      | [115]     |

PhIP-induced mutations in male and female gpt delta mice and reported that the highest MF was observed in the colon, followed by the spleen and liver. There were no gender differences in the MFs in the colon and liver. Stuart et al. [111] also examined the organ specificity of PhIP in Big Blue rats and reported that the MF in the colon was higher than that in the cecum and also that no gender differences were observed in the MFs in the colon.

IQ induces intestinal tumors and hepatocellular carcinoma but not in the kidney of rats [141–143]. Bol et al. [127] examined the mutagenicity of IQ in Big Blue rats and reported that the highest MF was observed in the liver, followed by the colon and kidney, a non-target organ. The higher MF in the liver than in the colon induced by IQ was also reported by Moller et al. [128]. MeIQ induces tumors in the Zymbal gland, oral cavity, colon, skin and mammary glands in F344 rats and tumors in the liver and forestomach of CDF1 mice [143]. Suzuki et al. [132] examined the mutagenicity of MeIQ in female Big Blue mice (C57BL/6N) and reported that the highest MF was in the colon, followed by the bone marrow, the liver and the forestomach. MeIQx induces liver tumors in CDF1 mice where the female mice are more susceptible than males, but does not induce tumors in the colon in both sexes [144]. Itoh et al. [135] examined the mutagenicity of MeIQx in Big Blue mice (C57BL/6) and reported that the MF in the liver was higher in female mice than in males. They also observed an increase in MFs in the colon, a non-target organ for carcinogenesis, where no obvious differences in MFs between male and female were observed. Mutagenicity in the colon of mice has also been reported in male gpt delta mice fed a diet containing MeIQx [136]. APNH is formed from aniline and norharman in vitro and in vivo and induces liver and colon cancers in F344 rats [145]. The in vivo mutagenicity of APNH was examined in male gpt delta mice fed a diet containing 10 or 20 ppm of APNH for 12 weeks [139]. The MF was higher in the liver than in the colon, and the MF in the liver of the mice at 20 ppm was almost equivalent to that of the liver in the same mice fed a diet containing 300 ppm MeIQx for 12 weeks [136]. ABAQ is a heterocyclic amine formed from glucose and L-tryptophan via the Maillard reaction. ABAQ has a tumor initiating-activity in the colon of mice [146]. The in vivo mutagenicity of ABAQ was examined in male gpt delta mice treated by gavage for 3 weeks at 25 or 50 mg/kg [140]. The MFs in the liver increased in a dose-dependent manner, and no MF was enhanced by the treatments in the kidney. AaC is the second most abundant heterocyclic amine in very well-done meat and fish [147]. It induces cancers in the liver and blood vessels of CDF1 mice [148]. The in vivo mutagenicity of AaC was examined in F1 (C57BL/6 x SWR) mice with lacI as a reporter gene [112]. AaC enhanced MFs in the colon but not in the small intestine.

**Mutation spectrum**

Mutagens induce specific types of sequence changes in the genome, such as T to C mutations by ethyl nitrosourea, G to T mutations by benzo[a]pyrene and CC to TT by ultraviolet light irradiation. DNA sequence changes associated with mutagenic treatments are called the “mutation spectrum”. In particular, specific sequence changes in cancer cells are called “mutational signatures,” which are important clues for investigating the causes of human cancer [149, 150]. PhIP induces colon cancer in male F344 rats where the adenomatous polyposis coli (Apc) gene is mutated by a guanine deletion at a 5’-GGGA-3’ [151]. Okonogi et al. [109] examined the mutation spectrum in the colon of Big Blue rats fed a diet containing PhIP and reported that one bp deletion was the most frequent mutation, including a guanine deletion at 5’-GGGA-3’ in male and female rats. Okochi et al. [116] investigated the mutation spectrum of mammary glands in female F1 (Big Blue rat x SD) rats...
administered 10 gavages of PhIP and concluded that G:C to T:A transversions were the most frequent mutations, followed by G:C deletions including G:C deletions at a 5′-GGGA-3′. Stuart et al. [119] examined the mutation spectrum in the prostate of Big Blue rats fed a diet containing PhIP and concluded that the predominant mutations were G:C to T:A transversions and deletions of G:C bp. In mice, Lynch et al. [114] treated Muta Mouse with PhIP and examined the mutation spectrum in the intestine. Approximately 40% of the total mutations were G:C to T:A transversions and 20% were G:C deletions, which were similar to those observed in the Hprt and DHFR genes in hamster and human cells in vitro. Okonogi et al. [115] examined the mutation spectrum of PhIP in the colon of Big Blue mice and reported that approximately half of the mutations were G:C to T:A transversions, in particular in runs of guanine, and approximately 1/4 of the total mutations were G:C deletions. In the colon, the rate of G:C to T:A transversions is significantly higher in mice than in rats [109]. Masumura et al. [117] treated male gpt delta mice with PhIP and reported that G:C to T:A transversions and G:C deletions in particular in 5′-TTTTTTG-3′ to 5′-TTTTTT-3′ were predominant mutations in the colon detected by gpt and SpI selections, respectively. Overall, it seems that PhIP induces G:C to T:A transversions and G:C deletions and that the transversions are more frequently induced in mice than in rats.

IQ predominantly induces G:C to T:A transversions in the liver of gpt delta rats and also in the liver and colon of Big Blue rats [127, 130]. G:C to T:A was also induced by MeIQ in the liver, bone marrow and colon of female Big Blue mice [115, 133], APNH in the liver and colon of male gpt delta mice [139] and AaC in the colon of Big Blue mice [115]. Mutational hot spots for G:C to T:A transversions by PhIP, MeIQ and AaC are in runs of guanine, at 5′-GC-3′ and in 5′-CGT-3′, respectively [115].

**No-observed effect level (NOEL) of in vivo mutagenesis**

Toxicological assays, including in vivo mutagenicity assays of chemicals, are conducted at high doses, i.e., the maximum tolerable doses (MTDs), which are often 1,000 or 10,000 times higher than the human exposure levels in daily life. Therefore, it is unclear whether the toxicity or mutagenicity observed at high doses can also be observed at low doses where humans are actually exposed to the chemical [152]. Lynch et al. [113] examined the mutagenicity of PhIP in Muta mice treated by oral gavage at doses of 0.2, 2 and 20 mg/kg for 4 days and reported that PhIP was mutagenic only at a dose of 20 mg/kg in the large intestine and liver. No mutagenicity was observed in the kidney, even at 20 mg/kg. They suggested that 2 mg/kg may be a potential threshold dose for PhIP-induced mutagenesis. They argued, however, that the dose may be a detection limit instead of a threshold because of the high spontaneous MFs in the liver of Muta mice. Gi et al. [131] examined the mutagenicity of IQ in male F344 gpt delta rats fed diets at doses of 0.1, 1, 10 or 100 ppm for 4 weeks and reported that gpt MFs were significantly enhanced over the control level at doses of 10 and 100 ppm but not at 0.1 and 1 ppm in the liver. They reported, however, that the frequencies of G:C to T:A transversions were significantly enhanced over the control level at a dose of 1 ppm in addition to 10 and 100 ppm and that the increase in the frequencies was dose-dependent. It suggests that DNA sequencing analysis may enhance the sensitivity of mutation detection, thereby lowering the no-observed-effect level (NOEL). Masumura et al. [136] examined the mutagenicity of MeIQx in male gpt delta mice fed diets containing MeIQx at doses of 3, 30 or 300 ppm for 12 weeks. The MFs in the liver significantly increased at doses of 30 and 300 ppm but not at 3 ppm. The frequency of G:C to T:A did not significantly increase at 3 ppm, either. In this case, DNA sequencing analysis did not affect the NOEL. Hoshi et al. [137] examined the mutagenicity of MeIQx in male F344 Big Blue rats fed diets at doses of 0.01, 0.1, 1, 10 or 100 ppm for 16 weeks and reported that the MFs significantly increased at 10 and 100 ppm in the liver. In addition, they examined glutathione S-transferase placental form (GST-P)-positive foci in the liver, which is a marker for hepatocarcinogenesis. The number of GST-P-positive foci significantly increased beyond the number of the control group only at a dose of 100 ppm. They suggested that the NOEL for in vivo mutagenesis was lower than that for carcinogenesis.

**Implication of in vitro and in vivo mutation assays**

The discovery of carcinogenic heterocyclic amines is one of the most fruitful scientific achievements enabled by the Ames test. Before this test was developed, the identification of chemical carcinogens solely depends on time-consuming animal tests. Multiple validation studies with more than 2,000 chemicals revealed that approximately 70-90% of chemical carcinogens are positive in the Ames test [153]. Therefore, this test is adopted in OECD test guideline 471 [154] and is widely used to eliminate potential carcinogens from pre-marketing chemicals developing for pharmaceuticals, pesticides, food additives and others. Owing to the power of the Ames test, it was initially expected that strong mutants in the Ames test might be strong carcinogens in rodents. However, studies with a large database indicated that the potency in the Ames test does not quantitatively correlate with that in rodent carcinogenicity assays [155]. The lack of quantitative relationships between mutagenesis in bacteria
and carcinogenesis in rodents may not be very surprising when considering the complex process of carcinogenesis such as mutation or initiation, promotion and progression. Since in vivo mutagenesis is much simpler than carcinogenesis, it was expected that the potency of the Ames test might correlate with that in transgenic mutation assays quantitatively. Although the mutagenic potency (revertants per μg) of MeIQ in strain TA98 is more than 300 times higher than that of PhIP [6], the MF of MeIQ in the colon of Big Blue mice fed a diet containing 300 ppm for 90 days is similar to that of PhIP in the mice fed a diet containing 400 ppm for 90 days [156]. It seems, therefore, that the mutagenic potency of the Ames test does not quantitatively correlate with the potency in in vivo mutation assays. It is also pointed out that the potency of the Ames test does not quantitatively correlate with that in in vitro mammalian cell assays for gene mutation and chromosome aberrations [153]. Despite the lack of quantitative correlations, the power of the Ames test to qualitatively predict potential carcinogens is outstanding, as evidenced by the successful discovery of carcinogenic heterocyclic amines.

Transgenic rodent mutation assays have enabled us to analyze chemical-induced mutations in various organs and tissues at the sequence level. Therefore, it would be interesting to examine whether we can predict target organs and sensitive gender for carcinogenesis based on the high MFs in specific organs and gender of rats and mice. Thus, the MFs were compared between the target organs and non-target organs for carcinogenesis, and the gender specificity in mice and rats was examined. However, the MFs in various lobes of the prostate were almost equally sensitive to the mutagenicity of PhIP, while the ventral prostate was the only target for cancer induction in rats [108, 125]. MeIQ induces much higher MF in the colon than in the liver, but the cancer incidence is higher in the liver than in the colon in mice [132, 157]. PhIP induces mutations in the colon of male and female rats, while colon cancer is induced only in males [107–109, 111]. MelIQx induces mutations in the colon of male and female mice, but it does not induce tumors in the colon [135, 136, 144]. These results indicate that target organs or tissues for carcinogenesis do not necessarily exhibit higher MFs compared to other organs or tissues, and also that mutations can be induced regardless of the gender specificity for carcinogenesis. In other words, the organs or tissues that are positive in the transgenic mutation assays are not necessarily carcinogenic targets. It appears, however, that tumors are induced in organs where mutations are induced when the carcinogens are genotoxic. Therefore, the transgenic mutation assays are employed to distinguish genotoxic carcinogens from non-genotoxic carcinogens [158]. The results of the transgenic mutation assays reflect in vivo metabolism and mammalian DNA repair, while the results of the Ames test reflect in vitro metabolism of S9 and bacterial DNA repair. Hence, the in vivo mutation assays may be useful to narrow down genotoxic carcinogens from chemicals that are positive in the Ames test. In fact, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) M7 for regulation of genotoxic impurities in pharmaceuticals recommends conducting in vivo mutation assays when the chemical is mutagenic in the Ames test [159]. Research on carcinogenic heterocyclic amines has provided valuable lessons on the effectiveness and limitations of in vivo transgenic mutation assays.

Since carcinogenic heterocyclic amines are produced by cooking, a question is whether they induce cancers in humans. If so, the extent to which they impose cancer risks on the general population? The exposure levels of heterocyclic amines are reported to be less than 500 ng per person per day [6]. In general, genotoxic carcinogens are regulated under the policy that they have no threshold or safe doses [152, 159, 160]. Therefore, there is carcinogenic risk to people who take carcinogenic heterocyclic amines. However, humans have various protective mechanisms against mutagenic substances such as detoxification, DNA repair, error-free translesion synthesis and apoptosis [161]. It is expected, therefore, that low-dose exposure to mutagenic carcinogens may be negligible due to these mechanisms. In addition, people are constantly exposed to endogenous mutagens such as reactive oxygen species. Thus, mutagenic risk is inevitable in humans. European Food Safety Authority (EFSA) and World Health Organization (WHO) propose 150 ng per person per day as a sufficient protective threshold of toxicological concern (TTC) for DNA-reactive genotoxic chemicals [162, 163]. Several studies with transgenic rodents exposed to low levels of carcinogenic heterocyclic amines have suggested the presence of NOEL [113, 131, 136, 137]. Although TTC is a concept that was developed to prioritize chemicals that require further toxicological evaluation and NOEL does not mean the absolute safe level, there may be certain exposure levels for genotoxic carcinogens, which do not increase excess lifetime cancer risk substantially. However, humans are exposed to multiple chemicals. Therefore, the combined risk should be evaluated. It has been reported that six carcinogenic heterocyclic amines, each of whose doses was below non-detectable levels by the Ames test, became mutagenic when they were combined [164]. In addition, chemicals may exhibit co-mutagenic effects and produce mutagenic substances when more than one non-mutagenic substance is combined [165]. Risk assessment of multiple exposures to DNA reactive mutagenic carcinogens at low levels may be a challenge that research on carcinogenic heterocyclic amines has proposed us.
Conclusions

Salmonella typhimurium YG strains help in the discovery of novel carcinogenic heterocyclic amines in complex mixtures such as food and river water by the Ames test because of the high sensitivity to mutagenic aromatic amines and nitroaromatics. Strain YG1024, which overproduces acetyltransferase, exhibited much higher sensitivity than TA98 for Glu-P-1, IQ, MelQ, MelQx and PBTA-1 but not for PhIP and Trp-P-2. It suggests that some of the heterocyclic amines are not activated by acetyltransferase. Transgenic rodent in vivo mutation assays are useful to analyze mutations in any organs of mice and rats at the sequence level. Heterocyclic amines induced tumors in the organs where mutations are induced. However, not all the organs where mutations are induced are target organs for carcinogenesis and the target organs for carcinogenesis are not necessarily organs where the highest MFs are observed. Research on carcinogenic heterocyclic amines provided valuable insights into the effectiveness and the limitation of in vitro and in vivo mutation assays for the identification of human carcinogens.

Abbreviations

IARC: International Agency for Research on Cancer; Trp-P-1: 3-amino-1,4-dimethyl-5H-pyrido [4,3-b]indole; Trp-P-2: 3-amino-1-methyl-5H-pyrido [4,3-b]indole; Glu-P-1: 2-amino-6-methylidipyrido [1,2-a:3,2′-d]imidazo[4-5-h]pyridine; Glu-P-2: 2-amino-6-methylidipyrido [1,2-a:3,2′-d]imidazo[4-5-h]pyridine; AaC: 2-amino-3-methyl-9H-pyrido [2,3-b]indole; MeAaC: 2-amino-3-methyl-9H-pyrido [2,3-b]indole; Salmonella typhimurium: Salmonella enterica subsp. enterica serovar Typhimurium; IQ: 2-Amino-3-methylimidazo [4,5-f]quinoline; MelQ: 2-amino-3,4-dimethylimidazo [4,5-f]quinoline; MelQx: 2-amino-3,8-dimethylimidazo [4,5-f]quinoline; PhiP: 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine; S9: 9000 x g supernatant of rat liver homogenates; mix: S9 plus an NADPH-generating system; PBTA-1: 2-(2-acylamino-4-(bis(2-methoxyethyl)amino)-5-methoxyphenyl)-5-amino-7-bromo-4-chloro-2-methoxyphenyl-norharman: 9-β-methylphenyl-9β-dimethyl-5H-pyrido [3,4-b]indole; ABAQ: 5-amino-6-hydroxy-8H-benzo [1,2] azepino [5,4-de]quinolin-7-one; APNH or aminophenylharman: 9-(4′-aminophenyl)-9H-pyrido [3,4-b]indole; N-OH-APNH or hydroxymethylphenylharman: 9-(4′-hydroxymethylphenyl)-9H-pyrido [3,4-b]indole; amino-2′-methylphenylharman: 9-(4′-amino-2′-methylphenyl)-9H-pyrido [3,4-b]indole; MF: mutant frequency; bps: base pairs; 6-TG: 6-thioguanine; SD: Sprague Dawley; F344: Fischer 344; Apc: adenomatous polyposis coli; MTD: maximum tolerable dose; NOEL: no observed effect level; GST-P: glutathione S-transferase placental form; ICH: International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; EFSA: European Food Safety Authority; WHO: World Health Organization; TTC: threshold of toxicological concern.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41021-021-00207-0.

Additional file 1.

Acknowledgements

We thank Dr. Kenichi Masumura, National Institute of Health Sciences, Kanagawa, Japan, for allowing us to cite the latest information about new homoyzogous gpr delta rats and for helpful comments on the manuscript. We also thank Professor Keiji Wakabayashi, Graduate School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Japan, for giving us permission to cite the structural formula of heterocyclic amines from reference [6]. We appreciate Professor Yukari Totsuka, School of Pharmacy, Nihon University, Chiba, Japan, for providing us the structural formula of APNH and ABAQ and Professor Tetsushi Watanabe, Department of Public Health, Kyoto Pharmaceutical University, Kyoto, Japan, for providing us the structural formula of PBTA1 and the related chemicals.

Authors’ contributions

MW and TN collected and analyzed published data. TN wrote the initial draft and MW edited the manuscript. The author(s) read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 3 June 2021 Accepted: 14 July 2021

Published online: 16 September 2021

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