Bacterial mutagenicity test data: collection by the task force of the Japan pharmaceutical manufacturers association

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Abstract

Background: Ames test is used worldwide for detecting the bacterial mutagenicity of chemicals. In silico analyses of bacterial mutagenicity have recently gained acceptance by regulatory agencies; however, current in silico models for prediction remain to be improved. The Japan Pharmaceutical Manufacturers Association (JPMA) organized a task force in 2017 in which eight Japanese pharmaceutical companies had participated. The purpose of this task force was to disclose a piece of pharmaceutical companies’ proprietary Ames test data.

Results: Ames test data for 99 chemicals of various chemical classes were collected for disclosure in this study. These chemicals are related to the manufacturing process of pharmaceutical drugs, including reagents, synthetic intermediates, and drug substances. The structure-activity (mutagenicity) relationships are discussed in relation to structural alerts for each chemical class. In addition, in silico analyses of these chemicals were conducted using a knowledge-based model of Derek Nexus (Derek) and a statistics-based model (GT1_BMUT module) of CASE Ultra. To calculate the effectiveness of these models, 89 chemicals for Derek and 54 chemicals for CASE Ultra were selected; major exclusions were the salt form of four chemicals that were tested both in the salt and free forms for both models, and 35 chemicals called “known” positives or negatives for CASE Ultra. For Derek, the sensitivity, specificity, and accuracy were 65% (15/23), 71% (47/66), and 70% (62/89), respectively. The sensitivity, specificity, and accuracy were 50% (6/12), 60% (25/42), and 57% (31/54) for CASE Ultra, respectively. The ratio of overall disagreement between the CASE Ultra “known” positives/negatives and the actual test results was 11% (4/35). In this study, 19 out of 28 mutagens (68%) were detected with TA100 and/or TA98, and 9 out of 28 mutagens (32%) were detected with either TA1535, TA1537, WP2uvrA, or their combination.

Conclusion: The Ames test data presented here will help avoid duplicated Ames testing in some cases, support duplicate testing in other cases, improve in silico models, and enhance our understanding of the mechanisms of mutagenesis.

Keywords: Ames test, Mutagenicity, Bacteria, In silico, Structure-activity relationship, Derek Nexus, CASE Ultra

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Introduction
The bacterial mutagenicity test, known as Ames test, is used worldwide to detect the mutagenicity of chemicals [1, 2]. Ames test is utilized not only for research purposes but also for submission to regulatory agencies for the approval of chemical substances [3, 4]. Recently, in silico evaluation of bacterial mutagenicity has been accepted by regulatory agencies [e.g., the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) M7 guideline [5] for hazard identification of mutagenic impurities in medicinal drugs]. In recent years, several in silico models for predicting bacterial mutagenicity have been developed. However, the prediction level is not fully satisfactory and remains to be improved [6–8]. One way to improve this is to collect Ames test data, particularly for chemicals in some chemical classes where a limited number of test data are available.

For this reason, the Japan Pharmaceutical Manufacturers Association (JPMA) organized a task force for Ames data sharing. The purpose of this task force was to disclose a piece of pharmaceutical companies’ proprietary Ames test data to make them available to anyone for utilization in research or submission to regulatory agencies, and to improve in silico models by using them as training set examples. Eight Japanese pharmaceutical companies participated in this task force, and Ames test data for 99 chemicals were collected. These chemicals are related to the manufacturing process of pharmaceutical drugs, including reagents, synthetic intermediates, and drug substances. In addition, in silico analyses of these chemicals for bacterial mutagenicity were conducted using a knowledge-based model (Derek Nexus, Lhasa Limited) or a statistics-based model (CASE Ultra, MultiCASE Inc.).

In this report, we present the Ames test data and in silico predictions for 99 chemicals of various chemical classes and discuss their structure-activity relationships in relation to structural alerts for each chemical class.

Materials and methods
Materials
Ninety-nine chemicals were tested and collected by this task force. Table 1 lists the chemical identification (ID), chemical name, CAS registry number (CAS No.), source, purity of the test chemicals used, and test site. Table 2 lists the chemical ID, chemical name (arranged by chemical classes), chemical structure, solvent used to dissolve the test chemicals, summarized Ames test results, and in silico analyses. In this study, free and salt forms were treated as different chemicals.

S9 fraction, prepared from the liver of phenobarbital/5,6-benzoflavone-pretreated male Sprague-Dawley rats, was purchased from Oriental Yeast (Tokyo, Japan) or Kikkoman Biochemifa (Chiba, Japan). The S9 mix consisted of 10% (v/v) S9 fraction (approximately 1.0 mg protein/plate), 8 mM MgCl₂, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADPH, 4 mM NADH, and 100 mM sodium phosphate (pH 7.4).

Bacterial strains
Four strains of Salmonella typhimurium, namely TA100, TA1535, TA98, and TA1537, and one strain of Escherichia coli, either WP2uvrA or WP2uvrA/pKM101 (for chemical IDs 21, 56, 58, 82, 93, and 94), were used in each Ames test. Chemical ID 57 was tested using only TA100, TA98, and WP2uvrA. These tester strains are recommended for use in bacterial mutagenicity test by the Organisation for Economic Cooperation and Development (OECD) test guideline 471 [3].

Ames test
All Ames tests were conducted using the preincubation method [9, 10]. Briefly, frozen stock cultures of each strain were inoculated into a conical flask or L-tube containing nutrient broth medium (2.5% w/v; Oxoid Nutrient Broth No.2, Hampshire, UK), and then cultured in a shaking incubator at 37 °C to obtain bacterial cells in the early stationary phase. The cell density of each culture was confirmed to be > 1 × 10⁹ cells/mL. For the tests carried out in the absence of S9 mix, 0.1 mL of the negative (vehicle) control solution, test chemical solution at various concentrations, or positive control solution was added to a test tube, to which 0.5 mL of 100 mM sodium phosphate buffer (pH 7.4) and 0.1 mL of bacterial culture were added. For the tests carried out in the presence of S9 mix, S9 mix was added in place of phosphate buffer. After mixing, the test tubes were preincubated at 37 °C for 20 min in a shaking water bath. After completion of the preincubation, the treatment mixture was immediately added and mixed with 2 mL of 0.05 mM L-histidine/0.05 mM D-biotin molten top agar (for Salmonella strains) or 0.05 mM L-trytophan (for E. coli strains), and the content was poured onto a plate of minimal-glucose agar medium. The plates were incubated at 37 °C for approximately 48 h, and the revertant colonies that appeared were counted. The sign of bacterial background lawn was examined as an indicator of cytotoxicity. In addition, the presence or absence of a precipitate of the test chemical was checked. When acetone, tetrahydrofuran, N,N-dimethylformamide, or 1,4-dioxane was used as the solvent, 0.05 mL of the vehicle was added to the test tube.

Multiple tests (dose-finding test, main test, or confirmatory test) were conducted for 86 chemicals. For 13 chemicals, a single test was conducted, and a clear conclusion was drawn. All tests were carried out in duplicate (two plates per dose) or triplicate (three plates per
| Chemical ID | Test chemical | CAS No. | Source or supplier of test chemical | Purity (%) | Test site |
|-------------|---------------|---------|------------------------------------|------------|----------|
| 1           | 1-Iodo-4-nitrobenzene | 636–98-6 | Maruzen Chemicals | 99.9 | CERI |
| 2           | 2-Nitro-5-(1-piperazinyl)benzaldehyde HCl | 1323630-2 | Otsuka Pharmaceutical | 100 | JISHA |
| 3           | Methyl 2-methyl-3-nitrobenzoate | 59382–59-1 | Otsuka Pharmaceutical | 99.57 | JISHA |
| 4           | 2-Nitro-5-(1-piperazinyl)benzaldehyde dimethyl acetal | 101291629-3 | Otsuka Pharmaceutical | 99.8 | JISHA |
| 5           | 5-Chloro-2-nitrobenzaldehyde dimethyl acetal | 13796–06-0 | Otsuka Pharmaceutical | 99.97 | JISHA |
| 6           | 2-Nitro-5-(1-piperazinyl)cinnamic acid | NR | Otsuka Pharmaceutical | 99.8 | JISHA |
| 7           | 2-Fluoro-4-nitrophenol | 403–19-0 | Tokyo Chemical Industry | 99.6 | BoZo Research Center |
| 8           | 3-Hydroxy-4-nitrobenzoic acid | 619–14-7 | Eisai | 99.6 | UBE |
| 9           | Pranidipine; Methyl (2E)-phenylprop-2-en-1-yl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate | 99522–79-9 | Otsuka Pharmaceutical | 99.97 | Otsuka Pharmaceutical |
| 10          | 4-Amino-2-fluorophenol | 399–96-2 | Tokyo Chemical Industry | 99.4 | BoZo Research Center |
| 11          | Methyl 3-amino-2-methyl benzoate | 18583–89-6 | Otsuka Pharmaceutical | 94.43 | JISHA |
| 12          | Sodium 3-[2-amino-5-(1-piperazinyl)phenyl]propionate | 101328646-3 | Otsuka Pharmaceutical | 99.5 | JISHA |
| 13          | Methyl 4-amino-2-methoxybenzoate | 27492–84-8 | Tokyo Chemical Industry | 98.9 | BoZo Research Center |
| 14          | Methyl 3-amino-4,6-dibromo-2-methylbenzoate | 119916–05-1 | Otsuka Pharmaceutical | 98.74 | JISHA |
| 15          | 4-(2-Methoxy-phenyl)-thiazol-2-ylamine | NR | Shionogi | 99.99 | CMIC Pharma Science |
| 16          | 4-Hexyl-1,3-thiazol-2-amine | 90770–58-4 | Shionogi | 99.72 | CMIC Pharma Science |
| 17          | 2-Amino-4-hydroxythiazole | 7146–26-1 | Oakwood Products | 89 | LSI Medience |
| 18          | Thiazole-2,4-diamine | 67355–26-4 | Oxchem | 98 | LSI Medience |
| 19          | 6-(2,3-Epoxypropoxy)-2(1H)-quinolinone | 143343–78-6 | Otsuka Pharmaceutical | 94.44 | JISHA |
| 20          | 6-(4-(3,4-Dimethoxybenzoyl)-2,3-dihydroxypiperezin-1-yl)-3,4-dihydriquinolin-2(1H)-one | NR | Otsuka Pharmaceutical | 98.12 | Otsuka Pharmaceutical |
| 21          | 8-Hydroxy-2(1H)-quinolinone | 15450–76-7 | Otsuka Pharmaceutical | 99.55 | JISHA |
| 22          | 3,4-Dimethoxy-N-[2,[(2-oxo-1,2,3,4-tetrahydroquinolin-6-yl)aminomethyl]benzamide | NR | Otsuka Pharmaceutical | 99.96 | Otsuka Pharmaceutical |
| 23          | 6-(3-Oxopiperezin-1-yl)-3,4-dihydroquinolin-2(1H)-one | NR | Otsuka Pharmaceutical | 99.56 | Otsuka Pharmaceutical |
| 24          | 3,4-Dihydro-5-(1-piperazinyl)-2(1H) quinolinone | 87154–95-8 | Otsuka Pharmaceutical | > 99.9 | JISHA |
| 25          | 6-(4-(4-Hydroxy-3-methoxybenzoyl)piperezin-1-yl)-3,4-dihydroquinolin-2(1H)-one | NR | Otsuka Pharmaceutical | 99.87 | Otsuka Pharmaceutical |
| 26          | 6-[1-Cyclohexyl-1H-tetrazol-5-yl]butoxy-2(1H)-quinolinone | 73963–62-9 | Otsuka Pharmaceutical | 100 | Otsuka Pharmaceutical |
| 27          | trans-3,4-Dihydro-6-[4-[1-(4-hydroxycyclohexyl)-1H-tetrazol-5-yl]butoxy]-2(1H)-quinolinone | 87153–04-6 | Otsuka Pharmaceutical | 99.93 | Otsuka Pharmaceutical |
| 28          | Grepafloxacin; (RS)-1-Cyclopropyl-6-fluoro-5-methyl-7-(3-methylpiperazin-1-yl)-4-oxo-quinoline-3-carboxylic acid | 119914–60-2 | Otsuka Pharmaceutical | 99.66 | JISHA |
| Chemical ID | Test chemical                                                                 | CAS No.   | Source or supplier of test chemical                  | Purity (%) | Test site   |
|------------|-------------------------------------------------------------------------------|-----------|------------------------------------------------------|------------|-------------|
| 29         | Grepafloxacin HCl; (RS)-1-Cyclopropyl-6-fluoro-5-methyl-7-(3-methylpiperazin-1-yl)-4-oxo-quinoline-3-carboxylic acid monohydrochloride | 161967–81–3 | Otsuka Pharmaceutical                               | 99.59      | Otsuka Pharmaceutical |
| 30         | Ethyl 1-cyclopropyl-7-bromo-6-fluoro-1,4-dihydro-5-methyl-4-oxo-3-quinolinecarboxylate | 119916–33–5 | Otsuka Pharmaceutical                               | 99.88      | JISHA       |
| 31         | 2,4-Bis(trimethylsiloxy)-5-fluoropyrimidine                                    | 17242–85–2 | Otsuka Pharmaceutical                               | 99.3       | JISHA       |
| 32         | 1,3-Dimethyl-2,4-pyrimidinedione                                                | 874–14–6  | Otsuka Pharmaceutical                               | 99.6       | JISHA       |
| 33         | 1-(Ethoxymethyl)-5-fluoro-pyrimidine-2,4-dione                                 | 57610–22–7 | Otsuka Pharmaceutical                               | 92.7       | JISHA       |
| 34         | 3-(3-Ethoxymethyl-5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-3-yl)carbonylbenzoic acid | 129971–17–1 | Otsuka Pharmaceutical                               | 99         | JISHA       |
| 35         | 3-(3-Benzoyloxycarbonylbenzyl)-1-ethoxymethyl-5-fluoro-2,4-pyrimidinedione     | NR        | Otsuka Pharmaceutical                               | 99.8       | JISHA       |
| 36         | 1-Hydroxybenzotriazole hydrate                                                | 123333–53–9 | Otsuka Chemical                                     | 99         | BML         |
| 37         | 3H-[1,2,3]Triazolo[4,5-b]pyridin-3-ol                                           | 39968–33–7 | Tokyo Chemical Industry                             | 99         | BML         |
| 38         | 1-[bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate | 148893–10–1 | Sigma-Aldrich                                      | 99         | BML         |
| 39         | Methylcarbamoyl-phenyloxadiazole                                              | 1374817–07–8 | Shionogi                                             | 98.77      | CERI        |
| 40         | 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate   | 3945–69–5  | Tokuyama                                            | 84.8       | BML         |
| 41         | N-Phenylbis(trifluoromethanesulfonylmide)                                     | 37595–74–7 | Tokyo Chemical Industry                             | 99.9       | SNBL        |
| 42         | 1,1,1-Trifluoro-N-phenylmethanesulfonamide                                     | 456–64–4   | Tokyo Chemical Industry                             | 99.9       | SNBL        |
| 43         | Perfluoro-1-butanesulfonyl fluoride                                            | 375–72–4   | Funakoshi                                           | > 90       | BML         |
| 44         | Diisopropyl sulfate                                                            | 2973–10–6  | Tokyo Chemical Industry                             | 97         | BML         |
| 45         | Methyl p-toluenesulfonate                                                      | 80–48–8    | Kanto Chemical                                     | 98         | BML         |
| 46         | Ethyl trifluoromethanesulfonate                                                | 425–75–2   | Tokyo Chemical Industry                             | 99.8       | SNBL        |
| 47         | 2-Nitrobenzenesulfonyl chloride                                                | 1694–92–4  | Mitsubishi Tanabe Pharma                             | 100.1      | Koei Techno |
| 48         | p-Toluenesulfonyl chloride                                                     | 98–59–9    | Tokyo Chemical Industry                             | 99         | BML         |
| 49         | 4,6-Dibromo-3-fluoro-2-methylbenzoyl chloride                                  | 11916–28–8  | Otsuka Pharmaceutical                               | 99.18      | JISHA       |
| 50         | Benzyl 3-chloroformylbenzoate                                                   | 67852–96–4  | Otsuka Pharmaceutical                               | 99.3       | JISHA       |
| 51         | 3-(1-Ethoxymethyl-5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-3-yl)carbonylbenzoic acid | 1380008–51–0 | Otsuka Pharmaceutical                               | 91.1       | JISHA       |
| 52         | 6-(3-Chloro-2-hydroxypropoxy)-2(1H)-quinolinone                                | 128669–85–8 | Otsuka Pharmaceutical                               | 95.18      | JISHA       |
| 53         | Chloroacetonitrile                                                             | 107–14–2   | Tokyo Chemical Industry                             | 99.9       | LSI Medience |
| 54         | 1-Bromohexane                                                                 | 111–25–1   | Tokyo Chemical Industry                             | 99.8       | BSRC        |
| 55         | 2-Chloro-N-methoxy-N-methylacetamide                                           | 67442–07–3  | Tokyo Chemical Industry                             | 99.9       | CMIC Pharma Science |
| Chemical ID | Test chemical | CAS No. | Source or supplier of test chemical | Purity (%) | Test site |
|-------------|---------------|---------|-------------------------------------|------------|----------|
| 56          | Ethyl 5-chloro-2-[2-(trifluoromethyl)phenyl]pentanimidate HCl | 1123197–78-3 | Eisai | 97.8 | UBE |
| 57          | Liothyronine sodium | 55–06-1 | Acros Organics | 95 | Taisho |
| 58          | (4-Bromo-3,5-dimethoxyphenyl)ethanol | 61367–62-2 | Eisai | 100 | UBE |
| 59          | Ethyl (4,6-dibromo-3-fluoro-2-methylbenzoyl)acetate | 119916–30-2 | Otsuka Pharmaceutical | 98.88 | JISHA |
| 60          | Catena-m-[2-ethoxycarbonyl-3-(4,6-dibromo-3-fluoro-2-methylphenyl)-3-oxidoacrylato(2-)-O,O′,O″′]-magnesium (II) | NR | Otsuka Pharmaceutical | 87.2 | JISHA |
| 61          | Methyl 4,6-dibromo-3-fluoro-2-methylbenzoate | 119916–08-4 | Otsuka Pharmaceutical | 99.72 | JISHA |
| 62          | 4,6-Dibromo-3-fluoro-2-methylbenzoic acid | 11916–27-7 | Otsuka Pharmaceutical | 98.79 | JISHA |
| 63          | Sodium 4,6-dibromo-3-fluoro-2-methylbenzoate | NR | Otsuka Pharmaceutical | 91.79 | JISHA |
| 64          | Ethyl 2-(4,6-dibromo-3-fluoro-2-methylbenzoyl)-3-cyclopropylaminopropenoate | NR | Otsuka Pharmaceutical | 99.95 | JISHA |
| 65          | Ethyl 2-(4,6-dibromo-3-fluoro-2-methylbenzoyl)-3-ethoxypropenoate | NR | Otsuka Pharmaceutical | 100 | JISHA |
| 66          | Cinnamyl 3-aminocrotonate | 113898–97-8 | Otsuka Pharmaceutical | 98.4 | JISHA |
| 67          | Cinnamyl acetoacetate | 57582–46-4 | Otsuka Pharmaceutical | 99.4 | JISHA |
| 68          | Benzyl hydrogen isophthalate | 113266–88-9 | Otsuka Pharmaceutical | 100 | JISHA |
| 69          | Sodium benzyl isophthalate | NR | Otsuka Pharmaceutical | 95.1 | JISHA |
| 70          | Dibenzyl isophthalate | 16034–14-3 | Otsuka Pharmaceutical | 99 | JISHA |
| 71          | Diethyl phosphoryl chloride | 814–49-3 | Tokyo Chemical Industry | 99 | BML |
| 72          | Bis(diphenylphosphino)ferrocene | 12150–46-8 | Hokko Chemical | 99.4 | BML |
| 73          | Phosphorus (III) bromide | 7789–60-8 | Tokyo Chemical Industry | 98 | BML |
| 74          | Triethyl phosphonoacetate | 867–13-0 | Tokyo Chemical Industry | 98.4 | BML |
| 75          | Dicyclohexyl(2′,6′-dimethoxybiphenyl-2-yl)phosphine | 657408–07-6 | Johnson Matthey | 100 | BML |
| 76          | 2-Dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (XPhos) | 564483–18-7 | Mitsubishi Tanabe Pharma | 99.87 | Koei Techno |
| 77          | Zinc cyanide | 557–21-1 | Alfa Aesar | 98.9 | BSRC |
| 78          | 3-Cyano-2,6-dihydroxypyridine monosodium salt | 91467–46-8 | Otsuka Pharmaceutical | 98.3 | JISHA |
| 79          | 3-Cyano-2,6-dihydroxypyridine | 35441–10-2 | Otsuka Pharmaceutical | 99.8 | JISHA |
| 80          | 6-Benzoyloxy-3-cyano-2-hydroxypyridine | 103941–70-4 | Otsuka Pharmaceutical | 100 | JISHA |
| 81          | Ethyl oxoacetate | 924–44-7 | Weylchem | 99.7 | FDSC |
| 82          | 2-Fluoro-3-hydroxy-5-methoxybenzaldehyde | 883576–31-6 | Eisai | 99.3 | UBE |
| 83          | 4-Bromobenzaldehyde | 1122–91-4 | Tokyo Chemical Industry | 99.9 | FDSC |
| 84          | 4-Pentyn-1-ol | 5390–04-5 | Avra Synthesis | 97.85 | LSI Medience |
dose), except for chemical ID 96 in dose-finding tests (single plate per dose). All solvents used were of high purity and were appropriate for use in Ames test.

Ames test data were generated in-house or in several Japanese contract research organizations in compliance with the Good Laboratory Practice (GLP), except for chemical IDs 47 and 57 (Table 1, Supplementary Tables).

Mutagenicity was evaluated according to the so-called “two-fold” rule [11]. The test chemical was judged to be positive (mutagenic) if the following criteria were satisfied: (1) the maximum number of revertants was two-fold or more relative to the negative (vehicle) control, (2) a dose-dependent increase in the number of revertants was observed, and (3) the results were reproducible between each test (if tests were conducted twice or thrice). Historical negative control counts in each laboratory were also considered for evaluation. Only chemical ID 4 was judged to be equivocal; although there was a clear dose-response relationship with reproducibility, the maximum number of revertants exceeded the upper limit of the historical negative control range, which was less than two-fold higher than the concurrent negative control counts.

In silico analyses
Chemicals were analyzed using a knowledge-based model [Derek Nexus (Derek), ver. 6.0.1; Lhasa Limited, Leeds, UK] and a statistics-based model (CASE Ultra, GT1_BMUT, ver. 1.8.0.2; MultiCASE Inc., OH, USA).

Results and discussion
The data for 99 chemicals, including four chemicals in the free and salt forms (chemical IDs 28 and 29, 62 and 63, 68 and 69, 78, and 79, respectively), were collected by the task force. The four pairs of these chemicals showed the same (negative) result with a similar toxicity between each pair, except for a pair of chemical IDs 28 and 29. Individual data are shown in Supplementary Tables. Table 2 lists the summarized Ames test and in silico analysis data of the test chemicals, which were arranged according to chemical classes. One-third of these chemicals were included in the training set for the latest

Table 1 Chemical ID, test chemical, CAS No. source or supplier of test chemical, purity, and test site (Continued)

| Chemical ID | Test chemical | CAS No. | Source or supplier of test chemical | Purity (%) | Test site |
|-------------|---------------|---------|-------------------------------------|------------|----------|
| 85          | (tert-Butoxycarbonyl)hydrazide | 870–46-2 | Shanghai Unibest Biopharma | 84.8 | BML |
| 86          | 4,6-Dibromo-3-methoxycarbonyl-2-methylbenzenediazonium tetrafluoroborate | NR | Otsuka Pharmaceutical | 93.2 | JISHA |
| 87          | 9-Fluorenylmethyl alcohol | 24324–17-2 | Tokyo Chemical Industry | 99.9 | BSRC |
| 88          | N-(3-Dimethylaminopropyl)-N\'ethyldimethylamine HCl | 25952–53-8 | Toyobo | 99 | BML |
| 89          | Benzamidoxime | 613–92-3 | Shionogi | > 98 | Koei Techno |
| 90          | Carboxothymethyl-dimethylsulfoxonium bromide | 5187-82-6 | Apollo Scientific | 96.5 | FDSC |
| 91          | 3,4-Dihydro-2H-pyran | 110–87-2 | Tokyo Chemical Industry | 99 | BML |
| 92          | (2S)-2-[(tert-Butoxycarbonyl)amino]hexanedioic acid dimethyl ester | 615258–01-0 | Eisai | 96.9 | UBE |
| 93          | tert-Butyl 2-acryloylhydrazine-1-carboxylate | 28689–14-7 | Eisai | 99.9 | UBE |
| 94          | [4-(Hydroxymethyl)-2,6-dimethoxyphenyl]boronic acid | 332394–37-3 | Eisai | 99.9 | UBE |
| 95          | Triethylsilane | 617–86-7 | Tokyo Chemical Industry | > 98 | BML |
| 96          | 1,3-Butanediol | 107–88-0 | Daicel | 99.8 | Nihon Bioreresearch |
| 97          | Ammonium acetate | 631–61-8 | Wako Pure Chemical Industries | 100 | CERI |
| 98          | p-Toluenesulfonic acid sodium salt | 7257–26-3 | Tokyo Chemical Industry | 99.7 | BML |
| 99          | 2,2,6,6-Tetramethylpipеридине 1-oxyl (free radical) | 2564–83-2 | Tokyo Chemical Industry | 99.7 | SNBL |

BSRC Biosafety Research Center, Foods, Drug and Pesticides, CERI Chemicals Evaluation and Research Institute, FDSC Hatano Research Institute, Food and Drug Safety Center, JISHA Japan Industrial Safety and Health Association, SNBL Shin Nippon Biomedical Laboratories, UBE UBE Scientific Analysis Laboratory, NR not registered

*PubChem Compound ID

*purified after purchase
Table 2 Chemical ID, chemical name, chemical structure, solvent used, Ames test result, and in silico analysis

| Chemical ID | Chemical name | Chemical structure | Solvent | Ames test result<sup>a</sup> | In silico analysis<sup>b</sup> |
|-------------|---------------|--------------------|---------|-----------------------------|-----------------------------|
| 1           | 1-Iodo-4-nitrobenzene | ![Chemical structure](image1) | DMSO    | Pos (-S9) in TA98, Pos (+S9) in TA100, TA1535 | Plausible (aromatic nitro compound) Positive |
| 2           | 2-Nitro-5-(1-piperazinyl)benzaldehyde HCl | ![Chemical structure](image2) | DMSO    | Pos (+S9) in TA100, TA98, WP2uvrA | Plausible (aromatic nitro compound) Positive |
| 3           | Methyl 2-methyl-3-nitrobenzoate | ![Chemical structure](image3) | DMSO    | Pos (+S9) in TA100 | Plausible (aromatic nitro compound) Positive |
| 4           | 2-Nitro-5-(1-piperazinyl)benzaldehyde dimethyl acetal | ![Chemical structure](image4) | DMSO    | Equivocal (+S9) in TA100 | Plausible (aromatic nitro compound) Positive |
| 5           | 5-Chloro-2-nitrobenzaldehyde dimethyl acetal | ![Chemical structure](image5) | DMSO    | Neg | Plausible (aromatic nitro compound) Positive |
| 6           | 2-Nitro-5-(1-piperazinyl)cinnamic acid | ![Chemical structure](image6) | DMSO    | Neg | Plausible (aromatic nitro compound) Positive |
| 7           | 2-Fluoro-4-nitrophenol | ![Chemical structure](image7) | DMSO    | Neg | Inactive Known Negative |
| 8           | 3-Hydroxy-4-nitrobenzoic acid | ![Chemical structure](image8) | DMSO    | Neg | Inactive Known Negative |
| 9           | Pranidipine | ![Chemical structure](image9) | DMSO    | Neg | Inactive Known Negative |
| 10          | 4-Amino-2-fluorophenol | ![Chemical structure](image10) | DMSO    | Pos (-S9) in TA100 | Inactive Known Positive |
| 11          | Methyl 3-amino-2-methyl benzoate | ![Chemical structure](image11) | DMSO    | Pos (+S9) in TA100, TA98 | Inactive Negative |
| 12          | Sodium 3-[2-amino-5-(1-piperazinyl)phenyl]propionate | ![Chemical structure](image12) | DMSO    | Neg | Plausible (aromatic amine or amide) Positive (as a free form) |
| 13          | Methyl 4-amino-2-methoxybenzoate | ![Chemical structure](image13) | DMSO    | Neg | Inactive Known Negative |
| 14          | Methyl 3-amino-4,6-dibromo-2-methoxybenzoate | ![Chemical structure](image14) | DMSO    | Neg | Inactive Inconclusive |
| 15          | 4-(2-Methoxy-phenyl)-thiazol-2-ylamine | ![Chemical structure](image15) | DMSO    | Pos (+S9) in TA100, TA98, TA1537, WP2uvrA | Plausible (aromatic amine or amide) Known Positive |
| 16          | 4-Hexyl-1,3-thiazol-2-amine | ![Chemical structure](image16) | DMSO    | Pos (+S9) in TA1535 | Plausible (aromatic amine or amide) Known Positive |
| 17          | 2-Amino-4-hydroxythiazole | ![Chemical structure](image17) | DMSO    | Neg | Plausible (aromatic amine or amide) Known Negative |
| 18          | Thiazole-2,4-diamine | ![Chemical structure](image18) | Distilled water | Neg | Plausible (aromatic amine or amide) Known Negative |
| 19          | 6-(2,3-Epoxypropoxy)-2(1H)-quinoxaline | ![Chemical structure](image19) | DMSO    | Pos (+S9) in TA100, TA1535, TA98, TA1537, WP2uvrA | Plausible (glycidyl ether, amine, ester or amide) Known Positive |
| 20          | 6-(4-(3,4-Dimethoxybenzoyl)-2,3-dihydroxy-piperazin-1-yl)-3,4-dihydroquinolin-2(1H)-one | ![Chemical structure](image20) | DMSO    | Pos (+S9) in TA100, TA1535, TA98, WP2uvrA | Inactive Negative |
| 21          | 8-Hydroxy-2(1H)-quinoxaline | ![Chemical structure](image21) | DMSO    | Pos (-S9) in TA1535 Pos (+S9) in TA1537 | Inactive Inconclusive |
| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|-----------------|-------------------|
| 22          | 3,4-Dimethoxy-N-{2-(2-oxo-1,2,3,4-tetrahydroquinolin-6-yl)aminooethyl}benzamide | ![Chemical Structure](image1) | DMSO         | Neg             | Inactive          | Positive          |
| 23          | 6-[3-Oxopiperazin-1-yl]-3,4-dihydroquinolin-2(1H)-one | ![Chemical Structure](image2) | DMSO         | Neg             | Inactive          | Negative          |
| 24          | 3,4-Dihydro-5-(1-piperazinyl)-2-(1H)quinoline | ![Chemical Structure](image3) | DMSO         | Neg             | Inactive          | Negative          |
| 25          | 6-(1-Cyclohexyl-1H-tetrazol-5-yl)butoxy-2(1H)-quinolinone | ![Chemical Structure](image4) | DMSO         | Neg             | Inactive          | Negative          |
| 26          | trans-3,4-Dihydro-6-[4-[1-(4-hydroxycyclohexyl)-1H-tetrazol-5-yl]butoxy]-2(1H)quinolinone | ![Chemical Structure](image5) | DMSO         | Neg             | Inactive          | Negative          |

**Fluoroquinolones**

| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|-----------------|-------------------|
| 28          | Grepafloxacin | ![Chemical Structure](image6) | DMSO         | Neg             | Plausible (quinolone-3-carboxylic acid or naphthyridine analogue) | Known Positive (as a free form) |
| 29          | Grepafloxacin HCl | ![Chemical Structure](image7) | Distilled water | Neg             | Plausible (quinolone-3-carboxylic acid or naphthyridine analogue) | Known Positive (as a free form) |
| 30          | Ethyl 1-cyclopropyl-7-bromo-6-fluoro-1,4-dihydro-5-methyl-4-oxo-3-quinolinecarboxylate | ![Chemical Structure](image8) | DMSO         | Neg             | Inactive          | Inconclusive      |

**Pyrimidinediones**

| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|-----------------|-------------------|
| 31          | 2,4-Bis(trimethylsiloxy)-5-fluoropyrimidine | ![Chemical Structure](image9) | DMSO         | Neg             | Inactive          | Out of Domain     |
| 32          | 1,3-Dimethyl-2,4-pyrimidinedione | ![Chemical Structure](image10) | Distilled water | Neg             | Inactive          | Negative          |
| 33          | 1-(Ethoxymethyl)-5-fluoro-pyrimidine-2,4-dione | ![Chemical Structure](image11) | DMSO         | Neg             | Inactive          | Negative          |
| 34          | 3-[1-Ethoxymethyl-5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-3-yl]carbonylbenzic acid | ![Chemical Structure](image12) | DMSO         | Neg             | Inactive          | Negative          |
| 35          | 3-[3-Benzoxycarbonylbenzoyl]1-ethoxymethyl-5-fluoro-2,4-pyrimidinedione | ![Chemical Structure](image13) | Acetone      | Neg             | Inactive          | Negative          |

**Triazoles**

| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|-----------------|-------------------|
| 36          | 1-Hydroxybenzotriazole hydrate | ![Chemical Structure](image14) | DMSO         | Neg             | Inactive          | Known Negative    |
| 37          | 3H-[1,2,3]Triazolo[4,5-b]pyridin-3-ol | ![Chemical Structure](image15) | DMSO         | Neg             | Inactive          | Negative          |
| 38          | 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate | ![Chemical Structure](image16) | DMF          | Neg             | Inactive (contains unclassified features) | Out of Domain |
| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|------------------|-------------------|
| 39 | Methylcarbamoyl-phenoxazine | ![Chemical Structure](image1) | DMSO | Neg | Inactive | Known Positive |
| 40 | 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate | ![Chemical Structure](image2) | Distilled water | Neg | Inactive | Out of Domain |
| 41 | N'-Phenylbis(trifluoromethanesulfonylimide) | ![Chemical Structure](image3) | DMSO | Neg | Inactive (contain unclassified feature) | Known Negative |
| 42 | 1,1,1-Trifluoro-N'-Phenylmethanesulphonamide | ![Chemical Structure](image4) | DMSO | Neg | Inactive | Known Negative |
| 43 | Perfluoro-1-butanesulfonyl fluoride | ![Chemical Structure](image5) | Acetone | Neg | Inactive | Known Negative |
| 44 | Disopropyl sulfate | ![Chemical Structure](image6) | 1,4-Dioxane | Pos (+S9) in TA100, TA1535, TA98, WP2uvrA | Plausible (alkylating agent) | Inconclusive |
| 45 | Methyl p-toluensulfonate | ![Chemical Structure](image7) | DMSO | Pos (+S9) in TA100, WP2uvrA | Plausible (alkylating agent) | Known Positive |
| 46 | Ethyl trifluoromethanesulfonate | ![Chemical Structure](image8) | DMSO | Neg | Plausible (alkylating agent) | Known Negative |
| 47 | 2-Nitrobenzenesulfonfyl chloride | ![Chemical Structure](image9) | DMSO | Pos (+S9) in TA100, TA98, Pos (+S9) in TA1535, TA98, WP2uvrA | Plausible (acid halide, aromatic nitro compound) | Known Negative |
| 48 | p-Toluensulfonfyl chloride | ![Chemical Structure](image10) | DMSO | Pos (+S9) in TA100, TA1535, TA98, WP2uvrA | Equivocal (acid halide) | Known Negative |
| 49 | 4,6-Dibromo-3-fluoro-2-methenylbenzoyl chloride | ![Chemical Structure](image11) | DMSO | Pos (-S9) in TA1535, WP2uvrA | Equivocal (acid halide) | Negative |
| 50 | Benzyl 3-chloroforamy benzoate | ![Chemical Structure](image12) | Acetone | Neg | | Equivocal (acid halide) | Negative |
| 51 | 3-(1-Ethoxymethyl-5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-3-yl)carbonylbenzoyl chloride | ![Chemical Structure](image13) | Acetone | Neg | | Equivocal (acid halide) | Negative |
| 52 | 6-(3-Chloro-2-hydroxypropoxy)-2(1H)-quinolinone | ![Chemical Structure](image14) | DMSO | Pos (-S9) in WP2uvrA, Pos (+S9) in TA100, TA1535, TA1537 | Plausible (alkylating agent) | Known Positive |
| 53 | Chloroacetontirile | ![Chemical Structure](image15) | DMSO | Pos (+S9) in WP2uvrA | Plausible (alkylating agent) | Known Positive |
| 54 | 1-Bromohexane | ![Chemical Structure](image16) | DMSO | Pos (+S9) in TA1535 | Plausible (alkylating agent) | Known Positive |
| 55 | Ethyl 5-chloro-2-(2-trifluoromethyl/phenyl)pentanimida te HCl | ![Chemical Structure](image17) | Distilled water | Neg | Plausible (alkylating agent) | Known Negative |
| 56 | Lithyronine sodium | ![Chemical Structure](image18) | DMSO | Neg (tested in TA100, TA98, WP2uvrA) | Inactive | Negative (as a free form) |
| 58 | (4-Bromo-3,5-dimethoxyphenyl)methanol | ![Chemical Structure](image19) | DMSO | Neg | Inactive | Known Negative |
### Table 2 Chemical ID, chemical name, chemical structure, solvent used, Ames test result, and in silico analysis (Continued)

| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|------------------|-------------------|
| 4,6-Dibromo-3-fluoro-2-methylbenzoates |
| 59 | Ethyl (4,6-dibromo-3-fluoro-2-methylbenzoyl)acetate | ![Structure Image] | DMSO | Pos (+S9) in TA100, TA1535, WP2uvA | Inactive | Known Positive |
| 60 | Catena-m(2-ethoxycarbonyl-3-(4,6-dibromo-3-fluoro-2-methylphenyl)-3-oxidoacrylato(2-)-O,O',O'',O''')magnesium(II) | ![Structure Image] | DMSO | Pos (+S9) in TA1535, WP2uvA | Not analyzed | Not analyzed |
| 61 | Methyl 4,6-dibromo-3-fluoro-2-methyl benzoate | ![Structure Image] | DMSO | Neg | Inactive | Negative |
| 62 | 4,6-Dibromo-3-fluoro-2-methylbenzoic acid | ![Structure Image] | DMSO | Neg | Inactive | Negative |
| 63 | Sodium 4,6-dibromo-3-fluoro-2-methylbenzoate | ![Structure Image] | DMSO | Neg | Inactive (as a free form) | Negative (as a free form) |
| 64 | Ethyl 2-(4,6-dibromo-3-fluoro-2-methyl benzoyl)-3-cyclopropylaminopropenoate | ![Structure Image] | DMSO | Neg | Inactive | Out of Domain |
| 65 | Ethyl 2-(4,6-dibromo-3-fluoro-2-methylbenzoyl)-3-ethoxypropenoate | ![Structure Image] | DMSO | Neg | Inactive | Negative |

**Cinnamyl alcohol esters**

| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|------------------|-------------------|
| 66 | Cinnamyl 3-aminoacetonate | ![Structure Image] | DMSO | Neg | Inactive | Negative |
| 67 | Cinnamyl Acetoacetate | ![Structure Image] | DMSO | Neg | Inactive | Negative |

**Benoates**

| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|------------------|-------------------|
| 68 | Benzyl hydrogen isophthalate | ![Structure Image] | DMSO | Neg | Inactive | Negative |
| 69 | Sodium benzyl isophthalate | ![Structure Image] | Distilled water | Neg | Inactive (as a free form) | Negative (as a free form) |

**Phosphorus-containing chemicals**

| Chemical ID | Chemical Name | Chemical Structure | Solvent Used | Ames Test Result | In Silico Analysis |
|-------------|---------------|--------------------|--------------|------------------|-------------------|
| 71 | Diethyl phosphoryl chloride | ![Structure Image] | THF | Pos (+S9) in TA98 | Inactive (contains unclassified features) | Negative |
| 72 | Bis(dihydroxophosphino)ferrocene | ![Structure Image] | 1,4-Dioxane | Neg | Inactive (contains unclassified features) | Out of Domain (evaluated without Fe²⁺) |
| 73 | Phosphorus(III) Bromide | ![Structure Image] | 1,4-Dioxane | Neg | Inactive (contains unclassified features) | Out of Domain |
| 74 | Triethyl phosphonoacetate | ![Structure Image] | DMSO | Neg | Inactive | Known Negative |
| 75 | Dicyclohexyl(2,6-dimethoxyphenyl-2-yl)phosphine | ![Structure Image] | THF | Neg | Inactive | Out of Domain |
| 76 | 2-Dicyclohexylyphosphino-2',6'-bis(trisopropylphenyl) (XPhos) | ![Structure Image] | DMSO | Neg | Inactive | Out of Domain |
Table 2 Chemical ID, chemical name, chemical structure, solvent used, Ames test result, and in silico analysis (Continued)

| Chemical ID | Chemical Name | Chemical Structure | Solvent | Ames Test Result | In silico Analysis |
|-------------|---------------|--------------------|---------|------------------|--------------------|
| 77          | Zinc cyanide  | ![Zinc Cyanide](image) | DMSO    | Neg              | Inactive (contains unclassified features) |
| 78          | 3-Cyano-2,6-dihydroxy pyridine monosodium salt | ![3-Cyano-2,6-dihydroxy pyridine monosodium salt](image) | DMSO    | Neg              | Inactive (as a free form) |
| 79          | 3-Cyano-2,6-dihydroxy pyridine | ![3-Cyano-2,6-dihydroxy pyridine](image) | DMSO    | Neg              | Out of Domain |
| 80          | 6-Benzoyloxy-3-cyano-2-hydroxy pyridine | ![6-Benzoyloxy-3-cyano-2-hydroxy pyridine](image) | DMSO    | Neg              | Inactive |

**Cyanides**

| Chemical ID | Chemical Name | Chemical Structure | Solvent | Ames Test Result | In silico Analysis |
|-------------|---------------|--------------------|---------|------------------|--------------------|
| 81          | Ethyl oxacetate | ![Ethyl oxacetate](image) | Acetone | Pos (+S9) in TA100, TA98, TA1535, WP2uvA | Positive |
| 82          | 2-Fluoro-3-cyano-5-methoxybenzaldehyde | ![2-Fluoro-3-cyano-5-methoxybenzaldehyde](image) | DMSO    | Neg              | Inactive |
| 83          | 4-Bromobenzaldehyde | ![4-Bromobenzaldehyde](image) | DMSO    | Neg              | Inactive |

**Alddehides**

| Chemical ID | Chemical Name | Chemical Structure | Solvent | Ames Test Result | In silico Analysis |
|-------------|---------------|--------------------|---------|------------------|--------------------|
| 84          | 4-Pentyn-1-ol | ![4-Pentyn-1-ol](image) | THF     | Neg              | Known Positive |
| 85          | (tert-Butylcarbonyl)hydrazide | ![tert-Butylcarbonyl)hydrazide](image) | THF     | Inactive         | Known Positive |
| 86          | 4,6-Dibromo-3-methoxybenzylidene-3-methylbenzeneidrazonium tetrafluoroborate | ![4,6-Dibromo-3-methoxybenzylidene-3-methylbenzeneidrazonium tetrafluoroborate](image) | DMSO    | Pos (+S9) in TA1535 | Positive |
| 87          | 9-Fluoromethyl alcohol | ![9-Fluoromethyl alcohol](image) | DMSO    | Pos (+S9) in TA1537 | Inactive |
| 88          | N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide HCl | ![N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide HCl](image) | DMSO    | Neg              | Known Positive |
| 89          | Benzenesulfonamide | ![Benzenesulfonamide](image) | DMSO    | Inactive         | Known Positive |
| 90          | 3,4-Dihydro-2H-pyran | ![3,4-Dihydro-2H-pyran](image) | Acetone | Neg              | Inactive |
| 91          | (E)-2-(tert-Butylcarbonyl)hexaneidoic acid dimethyl ester | ![E)-2-(tert-Butylcarbonyl)hexaneidoic acid dimethyl ester](image) | DMSO    | Neg              | Known Negative |
| 92          | tert-Butyl 2-acryloylhydrazine-1-carboxylate | ![tert-Butyl 2-acryloylhydrazine-1-carboxylate](image) | DMSO    | Neg              | Known Negative |
| 93          | [4-(Hydroxymethyl)-2,6-dimethoxyphenyl]boronic acid | ![4-(Hydroxymethyl)-2,6-dimethoxyphenyl]boronic acid](image) | Acetone | Neg              | Known Negative |

**Miscellaneous**

| Chemical ID | Chemical Name | Chemical Structure | Solvent | Ames Test Result | In silico Analysis |
|-------------|---------------|--------------------|---------|------------------|--------------------|
| 95          | Triethylsilane | ![Triethylsilane](image) | THF     | Neg              | Known Negative |
| 96          | 1,3-Butanediol | ![1,3-Butanediol](image) | THF     | Neg              | Inactive |
| 97          | Ammonium acetate | ![Ammonium acetate](image) | THF     | Neg              | Negative (as a free form) |
| 98          | p-Toluenesulfonic acid sodium salt | ![p-Toluenesulfonic acid sodium salt](image) | DMSO    | Neg              | Not analyzed |
| 99          | 2,2,6,6-Tetramethylpiperidine 1-oxide (free radical) | ![2,2,6,6-Tetramethylpiperidine 1-oxide](image) | DMSO    | Not analyzed     | Not analyzed |

The wording “Inactive” only indicates “inactive (negative)” call that does not contain “misclassified” or “unclassified” features.

“Out of Domain” fragments as well as “Inconclusive”, “Equivocal”, “Inactive (contains misclassified or unclassified features)” were treated as neither Ames-positive nor Ames-negative.

The wording “equivocal” used in Derek analysis is defined as the presence of an equal weight of evidence for and against the proposition.

The wording “Inactive” only indicates “inactive (negative)” call that does not contain “misclassified” or “unclassified” features.

“Out of Domain” fragments as well as “Inconclusive”, “Equivocal”, “Inactive (contains misclassified or unclassified features)” were treated as neither Ames-positive nor Ames-negative.

The wording “equivocal” used in Derek analysis is defined as the presence of an equal weight of evidence for and against the proposition.

The wording “Inactive” only indicates “inactive (negative)” call that does not contain “misclassified” or “unclassified” features.

“Out of Domain” fragments as well as “Inconclusive”, “Equivocal”, “Inactive (contains misclassified or unclassified features)” were treated as neither Ames-positive nor Ames-negative.

The wording “equivocal” used in Derek analysis is defined as the presence of an equal weight of evidence for and against the proposition.

The wording “Inactive” only indicates “inactive (negative)” call that does not contain “misclassified” or “unclassified” features.

“Out of Domain” fragments as well as “Inconclusive”, “Equivocal”, “Inactive (contains misclassified or unclassified features)” were treated as neither Ames-positive nor Ames-negative.

The wording “equivocal” used in Derek analysis is defined as the presence of an equal weight of evidence for and against the proposition.

The wording “Inactive” only indicates “inactive (negative)” call that does not contain “misclassified” or “unclassified” features.

“Out of Domain” fragments as well as “Inconclusive”, “Equivocal”, “Inactive (contains misclassified or unclassified features)” were treated as neither Ames-positive nor Ames-negative.

The wording “equivocal” used in Derek analysis is defined as the presence of an equal weight of evidence for and against the proposition.

The wording “Inactive” only indicates “inactive (negative)” call that does not contain “misclassified” or “unclassified” features.
version of CASE Ultra (where chemicals are presented as “Known positive” or “Known negative” in Table 2). The test chemicals were classified into the following chemical classes: nitrobenzenes, aromatic amines, 2-aminothiazoles, quinolinones, fluoroquinolones, pyrimidinediones, triazoles, heterocyclic compounds, sulfonyl derivatives, sulfonate esters, sulfonyl and benzoyl chlorides, halogenated alkanes, halogenated benzenes, 4,6-dibromo-3-fluoro-2-methylbenzoates, cinnamyl alcohol esters, benzoates, phosphorus-containing compounds, cyanides, aldehydes, and miscellaneous.

Structure-activity relationships
Although some chemical classes have only a few chemicals, we discuss the structure-activity (mutagenicity) relationships in relation to structural alerts.

Nitrobenzenes
The structure of nitroarenes is a representative alert for mutagenicity, although the simplest nitroarene nitrobenzene itself is not mutagenic [12–16]. All Ames-positive nitrobenzene derivatives were predicted to be mutagenic by both in silico models; however, in the present study, approximately half of the nitrobenzenes (5/9 chemicals) were non-mutagenic. The mutagenicity of nitroarenes can be generated through the reduction of the nitro moiety to the corresponding N-hydroxylamines by bacterial nitroreductase, and therefore can be efficiently detected in the absence of S9 mix [12–16]. Interestingly, chemical IDs 2–4 were mutagenic or equivocal only in the presence of S9 mix. One possible reason for nitrobenzene mutagenesis is the nitroreduction inside bacterial cells after oxidative metabolism in the S9 mix [15, 16].

Aromatic amines
The structure of aromatic amines is also a representative indicator of mutagenicity [12–14]. The primary mechanism of mutagenicity by aromatic amines is known to be the production of N-hydroxylation, typically by the CYP 1A2 enzyme, followed by O-esterification with acetate or sulfate [17, 18]. In this study, several aromatic amines (3/5 chemicals) were not mutagenic. Some substituents that generate electronic and/or steric effects probably inhibit mutagenicity through inhibition of drug-metabolizing enzymes involved and/or decreased stability of the nitrenium ion intermediate that was generated through cleavage of the N-O bond of esterified N-hydroxylamines and form adducts with DNA, leading to mutations [18, 19]. The mutagenicity of chemical ID 10 is probably due to reactive para-iminoquinone, which does not require metabolic enzymes.

2-Aminothiazoles
The 2-aminothiazoles tested, which were five-membered aromatic amines containing hetero atoms of sulfur in position 1 and nitrogen in position 3, were half mutagenic (2/4 chemicals) and half non-mutagenic (2/4 chemicals), with a diverse substituent at position 4. 2-Aminothiazoles were all predicted to be mutagenic (as “Plausible” by Derek) through identification of the structural alerts of aromatic amines or amidines. 2-Aminothiazole is mutagenic, and the mutagenicity of 2-aminothiazoles is induced via the formation of reactive nitrenium ion intermediates, such as aromatic amines [19–21]. The presence of a substituent at position 4 may enhance or reduce the mutagenicity of 2-aminothiazole.

Quinolinones
The six quinolinone derivatives (chemical IDs 22–27) were non-mutagenic, whereas the other three were mutagenic. The quinolinone structure was not an alert, as shown by both in silico models. Chemical ID 19 was mutagenic, probably because of the presence of epoxide. The mutagenicity of chemical ID 20 may be derived from the dihydroxylated piperazine moiety. Chemical ID 21, an 8-hydroxy derivative of quinolinone, was mutagenic only in TA1535, and TA1537, which shows a small number of negative control counts and is empirically known to be sensitive to some structures.

Fluoroquinolones
The mutagenicity of fluoroquinolones was dependent on WP2uvrA, WP2uvrA/pKM101, or TA102, which have an AT base pair at the primary reversion site [1–3]. Fluoroquinolone antibiotics, including grepafloxacin, were reported to be mutagenic in TA102 [22] and WP2uvrA/pKM101 [23], and the positive result was used as a training set in CASE Ultra. However, in this study, where WP2uvrA was used, the three fluoroquinolone derivatives, including grepafloxacin (chemical ID 28) and grepafloxacin HCl (chemical ID 29), were all non-mutagenic.

The difference of cytotoxicity (reduction in bacterial background lawn) in the two forms (chemical IDs 28 and 29) was much more than would be expected by normal variation. It may be worth looking at the role of the different solvents, including water and DMSO.

Pyrimidinediones
The five pyrimidinedione derivatives were all non-mutagenic. Both in silico models predicted these chemicals to be inactive/negative except for one chemical called the “Out of Domain” owing to the presence of two trimethylsilyl moieties, as shown by CASE Ultra. The structure of pyrimidinedione should not be an alert for mutagenicity.
**Triazoles**
All three triazole derivatives were non-mutagenic. Both in silico models predicted that these chemicals were inactive/negative except for the “Inactive containing unclassified features” and “Out of Domain” owing to the presence of a tertiary amine moiety, as shown by Derek and CASE Ultra, respectively. The structure of triazole is unlikely to be an indicator of mutagenicity.

**Heterocyclic compounds**
The two heterocyclic compounds, derivatives of oxadiazole (chemical ID 39) and 1,3,5-triazine (chemical ID 40), were both non-mutagenic. The finding that chemical ID 39 was non-mutagenic was not consistent with the “known positive” from CASE Ultra.

**Sulfonyl derivatives**
The three sulfonyl derivatives were all non-mutagenic, which was consistent with that in both in silico models, although Derek identified an unclassified feature of sulfonylimide in chemical ID 41. The structure of the sulfonyl moiety is not an alert for mutagenicity.

**Sulfonate esters**
Chemical IDs 44 and 45 were both mutagenic, and this result was consistent with the results of both in silico models. Several sulfonate esters are well-known to be alkylating mutagens, and predicted as “plausible” mutagens by Derek. However, chemical ID 46 was not mutagenic. The mutagenic potency of sulfonates is dependent on both the leaving group and alkylsulfonate moiety, affecting their chemical reaction rate [24, 25] and chemoselectivity [26, 27]. A probable reason for them being non-mutagenic is the rapid hydrolysis (instability) of ethyl trifluoromethanesulfonate [28]. The alertness of some sulfonate esters can be improved by incorporating the chemical properties.

**Sulfonyl and benzyol chlorides**
The two sulfonyl chlorides (chemical IDs 47 and 48) and benzyol chloride (chemical ID 49) were mutagenic in the presence or absence of S9 mix. Dimethyl sulfoxide (DMSO) was used as the solvent. It was reported that when DMSO was used to dissolve sulfonyl chlorides or acyl chlorides (including benzyol chlorides), these chemicals showed mutagenicity (or false positive results) due to the generation of mutagenic impurity (chlorodimethyl sulfide) in the test chemical formulations, with a few exceptions [29, 30]. Derek predicted sulfonyl and benzyol chlorides to be equivocal, the definition of which is that there is evidence for and against being mutagenic. These chemicals may not be mutagenic with organic solvents other than DMSO, such as acetone, where sulfonyl and acyl chlorides are stable. Water is probably not appropriate as a solvent, because these chemicals are generally unstable. Further tests on chemical IDs 47–49 are necessary to draw the correct conclusions. Nevertheless, the data presented here may be valuable as data examples when using solvents inappropriate for this chemical class. The other two benzyol chlorides, chemical IDs 50 and 51, were correctly judged to be non-mutagenic and dissolved in acetone.

**Halogenated alkanes**
Halogenated alkanes (halogen atoms excluding fluorine) can be alkylating mutagens without requiring metabolic activation. Similar to that of sulfonate esters, their mutagenic activity is dependent on the alkyl moieties and the leaving group of halogen ions. A possible reason why chemical IDs 55 and 56 were non-mutagenic is that the DNA adduct was not formed via inhibition of the SN2 reaction through steric hindrance by the bulky substituent. The carbon center adjacent to the chlorine atom. In this study, chemical ID 54 with a long alkyl chain (hexyl moiety) and a leaving group of bromine ions is marginally positive only in TA1535, which shows a low number of negative control counts in the presence of S9 mix, although n-butyl chloride with a shorter alkyl moiety is reported to be non-mutagenic [31]. Primary alkyl bromides with chains longer than the hexyl moiety are probably non-mutagenic.

**Halogenated benzenes**
The two halogenated benzenes were non-mutagenic. Chemical ID 57 was tested with three test strains, TA100, TA98, and WP2uvrA; the strains TA100 and TA98 were most sensitive among the five strains that are recommended for use by OECD test guideline 471 [3]. Halogenated benzenes are unlikely to be structural alerts for mutagenicity, as supported by Derek.

**4,6-Dibromo-3-fluoro-2-methylbenzoates**
Five 4,6-dibromo-3-fluoro-2-methylbenzoate derivatives (chemical IDs 61 to 65) were non-mutagenic, and Derek and CASE Ultra did not show alerts for this structure. Therefore, the structure of 4,6-dibromo-3-fluoro-2-methylbenzoate is not an indicator of mutagenicity. The mutagenicity of chemical ID 59 might involve the enol (tautomerized) form of the 1,3-diketone moiety, followed by epoxidation of the double bond by the drug-metabolizing enzyme in S9 mix. The substitution at position 2 of the 1,3-diketone moiety may inhibit tautomerization, but not lead to the induction of mutagenicity (chemical IDs 64, 65). It remains unclear why chemical ID 60 was mutagenic. Mutagenicity may be associated with the magnesium-oxygen complex.
**Cinnamyl alcohol esters**
Both cinnamyl esters were non-mutagenic, as predicted by both in silico models. A double bond conjugated with a benzene ring is unlikely to be a structural indicator of mutagenicity.

**Benzoates**
All benzoates were non-mutagenic, as predicted by both in silico models.

**Phosphorus-containing chemicals**
Phosphorus-containing chemicals were all non-mutagenic except for chemical ID 71, which is electrophilic and routinely used in organic synthesis for the phosphorylation of amines [32]. For many of the phosphorus-containing chemicals tested, neither of the in silico models were able to make a definite, positive/negative prediction; the reference to negative by Derek contained unclassified features, and CASE Ultra called “Out of Domain”. This indicates that phosphorus-containing chemicals are outside the applicability domain because of the limited number of training set examples for each in silico model.

*Cyanides*
Cyanide ion (Chemical ID 77) and all the cyanide derivatives substituted with an aromatic ring were non-mutagenic. The cyanide moiety is unlikely to be a structural alert for mutagenicity, as supported by Derek.

*Aldehydes*
Chemical ID 81, an aldehyde conjugated with a single carbonyl moiety, was mutagenic, as predicted by both in silico models. The chemical properties of aldehydes largely differ between aliphatic and aromatic compounds; generally, the former is chemically reactive, whereas the latter is stable. Both aromatic aldehydes (chemical IDs 82 and 83) were non-mutagenic, which can be explained by the extremely low chemical reactivity of aromatic aldehydes.

*Miscellaneous*
The miscellaneous group consists of chemicals that cannot be simply classified into the above chemical classes. Many of the chemicals tested were non-mutagenic. Chemical ID 84 was mutagenic in the presence and absence of S9 mix, although there were no structural alerts identified by Derek. The cause of the mutagenicity is unclear, but aldehyde might be involved in the induction of mutagenicity, which may be generated from alcohol by the alcohol dehydrogenase present in bacteria [33]. The three chemicals (chemical IDs 85–87) were mutagenic. Chemical IDs 85 and 86 were mutagenic only in WP2uvrA and TA1535, respectively. Both chemicals were predicted to be mutagenic (Derek; Plausible, CASE Ultra; Inconclusive or Positive) by both in silico models. Chemical ID 87 was only mutagenic in TA1537, which would be a tester strain sensitive to some chemical structures, with a small number of negative control counts.

**In silico analyses**
To calculate the sensitivity, specificity, and accuracy of in silico predictions, ten chemicals (chemical IDs 29, 47–49, 57, 60, 63, 69, 78, and 99) were excluded. Four chemicals tested in both forms were used for calculation in the free form (chemical IDs 28, 62, 68, and 79), but not in the salt form (chemical IDs 29, 63, 69, and 78). Chemical IDs 47–49 were false positive because probable inappropriate solvents were used. Chemical ID 57 was tested in only three strains (TA100, TA98 and WP2uvrA). For chemical IDs 60 and 99, the in silico models could not reach a conclusion because the former is a complex molecule and the latter is a radical. We treated “Out of Domain” fragments as well as “Inconclusive”, “Equivocal”, “Inactive (contains misclassified or unclassified features)”, as neither Ames-positive nor Ames-negative in this study.

In silico analysis using Derek (ver. 6.0.1) revealed the sensitivity, specificity, and accuracy to be 65% (15/23), 71% (47/66), and 70% (62/89), respectively. In contrast, in silico analysis using CASE Ultra (GT1_BMUT, ver. 1.8.0.2) revealed the sensitivity, specificity, and accuracy to be 50% (6/12), 60% (25/42), and 57% (31/54), respectively. Thus, Derek outperformed CASE Ultra (GT1_BMUT) in the predictive level of bacterial mutagenicity for all the parameters in this study, where the limited number of chemicals were compared.

Derek and Case Ultra occasionally called “inactive containing misclassified or unclassified features” (8 chemicals), and “Out of Domain” fragments (10 chemicals), respectively, indicating the need to expand the training or reference set for each in silico model to improve.

It is worth noting that when considering the performance of the in silico models, it is important to account for the ICH M7 approach of combining two complementary systems and an expert review to take a final decision rather than considering them separately [5, 34].

**Inconsistency with training set examples**
The 35 chemicals (15 “known” positives and 20 “known” negatives) were part of the training set for CASE Ultra. The results for 4 of 35 chemicals (11%) did not agree with the known response for those chemicals in that training set. The four chemicals (chemical IDs 28, 39, 88, and 89) were non-mutagenic but were registered as
mutagens in the training set for CASE Ultra. This disagreement ratio (11%) was in the same range as the Ames test non-reproducibility, identified by Piegorsch and Zeiger, who reported a value of approximately 13% [35]. The reasons why the Ames test evaluations did not match were mainly some differences in the test conditions (e.g., plate-incorporation method vs. preincubation method, the type of strains used, source of test strains, preparation of overnight culture), and evaluation criteria (e.g., two-fold rule vs. statistical analysis), and quality of test substances [10, 11, 36].

Two chemicals (chemical IDs 47 and 48) were mutagenic but were registered as non-mutagens in the CASE Ultra training set. This is probably because the solvent used in our study was not appropriate, as previously stated (see the section of “Sulfonyl and benzoyl chlorides” in the Structure-activity relationships section. Our data, together with individual data (Supplementary Tables), provide additional information and will help in reevaluating the Ames test data.

Test strains to detect bacterial mutagens
In this study, 28 chemicals, including three sulfonyl and benzoyl chlorides (chemical IDs 47 to 49) were mutagenic. Among them, three chemicals (chemical IDs 16, 54, and 86), two chemicals (chemical IDs 21 and 87), two chemicals (chemical IDs 53 and 85), and two chemicals (chemical IDs 49 and 60), respectively, were only detected for mutagenicity in either TA1535, TA1537, and WP2uvrA, or both TA1535 and WP2uvrA. Williams et al. [36] reported that 93% of bacterial mutagens can be detected with a combination of TA100 and TA98. However, the data of this present study show that only 19 out of 28 chemicals (68%) were detected either by TA100 or TA98. Therefore, the test strains TA1535, TA1537, and WP2uvrA may be useful for the efficient detection of bacterial mutagenicity.

Conclusion
Ames test data were presented for 99 chemicals from eight pharmaceutical companies through the activity of the Ames data sharing task force. The chemicals were related to the manufacturing process of pharmaceutical drugs, including reagents, synthetic intermediates, and drug substances. The Ames test data presented herein will contribute to avoiding duplicated Ames test in some cases, supporting duplicate testing in other cases, improving in silico models, and enhancing our understanding of the mechanisms of mutagenesis.

Abbreviations
9AA: 9-aminopurine; 2AA: 2-aminoanthracene; B(6)P: Benzo[a]pyrene; ICR-191: 6-chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxyacridine dihydrochloride; DMSO: Dimethyl sulfoxide; AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; GLP: Good Laboratory Practice; ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; JPMA: Japan Pharmaceutical Manufacturers Association; OECD: Organisation for Economic Cooperation and Development

Supplementary Information
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Additional file 1.

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Authors’ contributions
AH analyzed the chemicals using Derek and drafted and edited the manuscript. AH, TA, TS, AO, MY, KK, HO, YD, SO, KS, TK, and EY prepared the Supplementary Tables and reviewed the manuscript. All authors approved the final manuscript.

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Availability of data and materials
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