Recommended Follow-Up Testing for an Ames-Positive Drug (Active Ingredient) or Metabolite To Support First-in-Human Clinical Trials With Healthy Subjects

Guidance for Industry and Review Staff

DRAFT GUIDANCE

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U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)

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Pharmacology/Toxicology

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Recommended Follow-Up Testing for an Ames-Positive Drug (Active Ingredient) or Metabolite To Support First-in-Human Clinical Trials With Healthy Subjects Guidance for Industry and Review Staff[1]

This draft guidance, when finalized, will represent the current thinking of the Food and Drug Administration (FDA or Agency) on this topic. It does not establish any rights for any person and is not binding on FDA or the public. You can use an alternative approach if it satisfies the requirements of the applicable statutes and regulations. To discuss an alternative approach, contact the FDA staff responsible for this guidance as listed on the title page.

I. INTRODUCTION

The purpose of this guidance is to inform industry and the review staff in the Center for Drug Evaluation and Research (CDER) on how CDER views positive findings in the in vitro bacterial reverse mutation (Ames) test of a drug (active ingredient) or its metabolites and to provide recommendations on follow-up in vitro and in vivo mutagenicity testing of Ames-positive active ingredients to support the enrollment of healthy human subjects in first-in-human (FIH) clinical trials.

This guidance generally pertains to active ingredients of certain drug products intended to be submitted for approval under section 505 of the Federal Food, Drug, and Cosmetic Act[2] administered by all clinical routes. The focus of this guidance is testing of new small molecule active ingredients in healthy human subjects in FIH trials. It does not apply to (1) biological products intended to be submitted for licensure under section 351 of the Public Health Service Act,[3] (2) active ingredients of drug products intended to treat patients with advanced cancer, or (3) DNA reactive (mutagenic) impurities.[4]

[1] This guidance has been prepared by the Genetic Toxicology Subcommittee as directed by the Pharmacology Toxicology Coordinating Committee in the Office of New Drugs in the Center for Drug Evaluation and Research at the Food and Drug Administration.

[2] See 21 U.S.C. 355.

[3] See 42 U.S.C. 262.

[4] See the ICH guidance for industry *M7(R2) Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk* (July 2023). We update guidances periodically. For the most recent version of a guidance, check the FDA guidance web page at https://www.fda.gov/regulatory-information/search-fda-guidance-documents.

In general, FDA's guidance documents do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

The timing and conduct of genetic toxicology studies for assessing the safety of an active ingredient are described in the ICH guidances for industry *M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals* (January 2010), *S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use* (June 2012), and *S9 Nonclinical Evaluation for Anticancer Pharmaceuticals* (March 2010). We recommend referring to these guidances, with this document supplementing the recommendations in those guidances.

Genotoxicity tests can be defined as in vitro and in vivo tests designed to identify compounds that induce genetic damage (mutagenicity or clastogenicity) by various mechanisms.[5] These tests enable hazard identification with respect to DNA damage and its fixation. Fixation of DNA damage is the process by which gene mutations (i.e., changes in DNA sequence that affect a single gene) and larger scale alterations, such as chromosome loss or translocations, all of which are considered irreversible effects, become established in the cell. These changes are potentially heritable and cancer-causing. Genetic alterations, however, are only one factor responsible for cancer. Cancer is viewed as the outcome of a complex, multistep process involving genetic alterations, possibly in combination with nongenetic determinants.

Genotoxicity tests play a significant role in protecting clinical trial subjects from potential increased risk of genotoxic hazard and cancer during the investigational new drug application (IND) phase of drug development. A standard battery of genetic toxicology studies has been accepted by industry and regulators through the ICH consultative process.[6]

As described in ICH M3(R2), a standard battery of tests measuring mutagenicity and other manifestations of genetic damage (Option 1 or 2)[7] is often conducted before the initiation of phase 1 clinical trials to protect human subjects and is recommended to be completed before the start of phase 2 trials conducted in healthy subjects and patients with the disease or condition the investigational new drug containing the active ingredient of interest is intended to treat (active ingredients being developed for oncology indications should follow recommendations in ICH S9 regarding genotoxicity testing). The carcinogenic potential of an active ingredient, usually determined in rodent bioassays (i.e., a 2-year mouse or 6-month rasH2 mouse carcinogenicity

[5] See ICH S2(R1).

[6] See ICH S2(R1).

[7] See ICH S2 (R1)

study and 2-year rat study (a weight-of-evidence (WoE) carcinogenicity risk assessment might suffice in lieu of a 2-year rat study under appropriate circumstances)), is typically not known until late in development, often when phase 3 trials are near completion or have been completed, or at the time of intended submission of the new drug application.[8]

Generally, most active ingredients found to be positive for mutagenicity (i.e., Ames-positive, the only test in both Options 1 and 2 of the standard battery that specifically assesses mutagenicity) are not further developed for approval by FDA. Due to their therapeutic mechanism of action, active ingredients used for treating oncology indications may be an exception and might be further developed for potential approval by FDA, even if they are mutagenic. Although ICH S2(R1) provides recommendations for follow-up studies for positive in vitro mammalian cell genotoxicity assays (i.e., assays primarily intended to detect clastogenicity and/or aneugenicity), no specific guidance is provided in that ICH guideline for follow-up testing of an Ames-positive (mutagenic) active ingredient. Positive results in the Ames test suggest potential DNA reactivity, indicating that follow-up testing and evaluation of an Ames-positive active ingredient are necessary to assess its in vivo mutagenic and carcinogenic potential.[9] Positive results in the Ames test are correlated with carcinogenic potential in rodents;[10] however, this correlation is not perfect because mutations are only one of many stages in tumor development. In addition, the mutagenic response may be due to exceeding a detoxification threshold or the induction of oxidative damage to which bacterial cells may be more sensitive than mammalian cells in vitro or tissues in vivo.

This guidance makes recommendations on follow-up testing for Ames-positive active ingredients in those rare circumstances when a sponsor decides to continue development. These recommendations are intended to potentially address and lower certain safety concerns before proceeding with FIH trials in healthy human subjects. Follow-up testing cannot entirely mitigate the concerns raised by an Ames-positive finding, and some residual risk remains in the absence of an adequate carcinogenicity assessment. Thus, Ames-positive active ingredients that are further developed should be those targeting serious or life-threatening diseases with unmet medical needs.

Healthy human subjects are commonly enrolled in phase 1 FIH trials of the investigational new drug containing the active ingredient of interest. These studies are typically of short duration (up to 2 weeks) and involve close monitoring. The administration of doses may be continuous or intermittent (e.g., having a washout period of several half-lives between doses). Healthy subjects receive no direct treatment benefit from trial participation. Thus, a robust nonclinical program of studies that can help characterize potential risk to determine that it is sufficiently low is required before FIH trials can commence in healthy human subjects.

[8] See the ICH guidances for industry *S1A The Need for Long-term Rodent Carcinogenicity Studies of Pharmaceuticals* (March 1996), *S1B Testing for Carcinogenicity of Pharmaceuticals* (July 1997), *S1B(R1) Addendum to S1B Testing for Carcinogenicity of Pharmaceuticals* (November 2022), and *S1C(R2) Dose Selection for Carcinogenicity Studies* (September 2008).

[9] See ICH S2(R1).

[10] See Note 2.

Patients with the disease or condition, who may receive a treatment benefit with an investigational new drug, usually are enrolled (1) when longer duration phase 1, 2, and/or 3 trials are conducted or (2) in trials for an investigational new drug that possesses a safety profile not appropriate for healthy subjects.

Significant safety concerns have been raised regarding whether it is appropriate to administer even one dose of an Ames-positive active ingredient to healthy subjects. Even with a single dose, there is a nonzero probability of increasing the subject's cancer risk.[11] This topic was discussed in an FDA Genetic Toxicology Workshop[12] held on November 4, 2019.

Consideration might be given to the administration of an Ames-positive active ingredient to healthy human subjects only if the results of extensive follow-up testing (refer to the decision tree in Figure 1 of this guidance) conducted before clinical administration lowered the concern for cancer based on a WoE approach evaluating the potential for mutagenicity. A WoE evaluation, for instance, might find that follow-up testing in an in vitro mammalian cell mutation assay and in vivo mutation assay is negative and that other considerations described below do not raise any other safety concerns (refer to decision tree in Figure 1). An Ames-positive metabolite observed at low levels (e.g., at the threshold of toxicological concern) would generally pose minimal safety concerns and may be managed differently. Along with negative results in the in vitro and in vivo assays, the sponsor also should provide a thoroughly considered rationale for why FIH trials should enroll healthy subjects in lieu of patients with the disease or condition. Positive findings in either the in vitro mammalian cell mutation assay or the in vivo mutation assay would preclude FIH trials in healthy subjects. Alternatively, consideration should be given to enrolling patients with the disease or condition of interest and designing the study in a manner that offers the prospect of direct treatment benefit in addition to the usual aims of a phase 1 trial (e.g., pharmacokinetics, tolerability, etc.).

If testing is considered, a consistent process of follow-up testing and evaluation should first be conducted for an Ames-positive active ingredient (refer to the decision tree in Figure 1) before commencing FIH trials in healthy subjects. Early consultation with the relevant CDER review division through the pre-IND process is strongly recommended before submission of an IND that proposes conducting a FIH clinical trial in healthy subjects with an Ames-positive active ingredient. These recommendations for follow-up testing are intended to inform both review staff and industry.

[11] See ICH M3(R2). Also see Note 1.

[12] See "How Many Doses of an DNA Reactive (Ames-positive) Drug Can Be Safely Administered to Healthy Subjects?" available at https://www.fda.gov/news-events/fda-meetings-conferences-and-workshops/fda-genetic-toxicology-workshop-how-many-doses-dna-reactive-ames-positive-drug-can-be-safely.

III. RECOMMENDED ANALYSIS OF AMES TEST DATA AND FOLLOW-UP TESTING FOR AN AMES-POSITIVE ACTIVE INGREDIENT OR METABOLITE

If the Ames test results indicate a potential human mutagenic risk (see section III.A), and no other mutagenicity data are available, follow-up mutagenicity testing, first with in vitro assays (see section III.B) and then with in vivo assays (see section III.C), should be conducted to assess the relevance of the Ames-positive result. Specific considerations for testing metabolites are outlined in section III.D, and the need for carcinogenicity testing is discussed in section III.E.

A. Evaluation of the Ames Test Before Follow-Up Assessments[13]

There are several criteria that should be considered in the evaluation of an Ames test response with an active ingredient or its metabolites. These include use of standardized criteria, identification of the mutagenic structural alert with a positive response, and various factors that can influence the test results. Refer to the decision tree in Figure 1 of this guidance that outlines the criteria that should be considered in the evaluation of an Ames test response.

- The Ames test serves as the primary assessment of the mutagenic potential for an active ingredient candidate. The test is conducted following standard methods as described in the Organisation for Economic Cooperation and Development (OECD) Test Guideline (TG) 471 (OECD 2020).

- To maximize the value of data from the test, results from the Ames test should be evaluated using the criteria described by Levy et al. (2019).

- In addition, evaluation of an Ames-positive finding for potential mitigating factors should be conducted.[14]

  - The functional group of the molecule responsible for the Ames-positive finding could be compared through read-across to chemicals with a similar functional group that have available carcinogenicity data. Such information may increase or decrease the level of concern with respect to carcinogenic potential.

  - If applicable, the possibility of a bacterial-specific positive response (e.g., due to bacterial-specific metabolism (nitro reduction)) or a positive response not caused by mutation (e.g., due to the presence of free histidine or tryptophan) lessens the relevance of the Ames-positive finding for in vivo mutation.

  - If any human data are available, comparability of metabolic profiles in rodents and humans would be informative. A positive finding that could be linked to a rodent-specific metabolism that is not relevant to humans would lessen the concern for

[13] See decision tree, box 1.

[14] See decision tree, box 5.

conducting a clinical trial. Under some circumstances, it may be necessary to synthesize the metabolite and test it using in vitro and in vivo follow-up tests in a similar manner as was done for the parent active ingredient (see section III.D).

B. Follow-Up Testing of an Ames-Positive Active Ingredient With an In Vitro MLA or Mammalian Cell HPRT Assay[15]

The initial follow-up testing should be conducted in the mouse lymphoma assay (MLA) or in a mammalian cell hypoxanthine-guanine phosphoribosyl transferase (HPRT) forward mutation test, as they possess high sensitivity to detect mutagenic events. Both tests detect small-scale mutations similar to the mutations detected by the Ames test (OECD 2016a; OECD 2016b). The MLA, however, also detects aneugenicity and clastogenicity (OECD 2016a), and MLA data should be analyzed with the objective of evaluating smaller scale gene mutations. Support for use of the in vitro MLA or mammalian cell HPRT test as follow-up to further evaluate an Ames-positive in vivo mutagen was provided by Kirkland et al. (2014).[16] Justification should be provided for the test selection. An MLA or a HPRT test that has been conducted as part of the standard battery of genotoxicity tests (decision tree, box 2) could be used in lieu of conducting a new test.

For the MLA, colony sizing should be conducted. Large colony mutants would be evidence of a mutagenic event consistent with Ames-positive findings (OECD 2016a).

C. Follow-Up Testing of an Ames-Positive Active Ingredient With the Transgenic Rodent Gene Mutation Assay and/or *Pig-a* Gene Mutation Assay[17]

An in vivo mutation assay is generally recommended for an Ames-positive active ingredient that was negative in the MLA or HPRT forward mutation test (decision tree, box 9). Under appropriate circumstances, there might be a need to determine the in vivo mutagenic response under more relevant physiological conditions. As the influence of absorption, distribution, metabolism, and excretion (ADME) factors may be missing or highly altered in in vitro tests with bacteria or mammalian cells, in vivo testing is important for understanding the relevance of a positive Ames test (Lambert et al. 2005; Nohmi et al. 2017; OECD 2022a; OECD 2022b). An in vivo test could be helpful in a WoE decision (i.e., evaluation of all available data) in the determination of whether it is reasonably safe to proceed with FIH clinical trials and if inclusion of healthy subjects is appropriate. The transgenic rodent (TGR) gene mutation assay (OECD 2022b) and/or *Pig-a* gene mutation assay (OECD 2022a) are acceptable, with appropriate justification, to assess the relevance of a positive in vitro Ames test for in vivo mutation. These assays, like the in vitro Ames test, can detect small-scale genetic damage that may be caused by mispairing or misincorporation of bases during replication, as well as small DNA sequence

[15] See decision tree, box 7.

[16] See Note 3.

[17] See decision tree, box 9.

additions, deletions, or rearrangements (Lambert et al. 2005; Nohmi et al. 2017; OECD 2022a; OECD 2022b).

- The TGR gene mutation assay is recommended to evaluate in vivo mutagenicity in multiple tissues (Lambert et al. 2005; Nohmi et al. 2017; OECD 2022b). It could be advantageous to include a *Pig-a* endpoint (2022a) in the assay because if the *Pig-a* endpoint is positive, then the tissue analysis would not be needed (Robison et al. 2021). However, if the *Pig-a* endpoint assay was negative, tissue analysis for transgene mutation should proceed.

- There could be circumstances, with appropriate justification, when a 28-day *Pig-a* assay would be sufficient and a TGR mutation study is not needed. The bone marrow, which serves as the target tissue for detecting *Pig-a* mutation, is generally regarded as a rapidly dividing and well-perfused tissue (OECD 2022a). There may be circumstances based upon justification from ADME and general toxicology data (e.g., histopathology, bone marrow smear evaluation, tissue selective response appears unlikely, high plasma active ingredient exposure and low tissue exposures, etc.) under which conducting a *Pig-a* assay alone (without the TGR mutation assay) could be acceptable.

- OECD TG 488 (2022b) and 470 (2022a) provide details on the appropriate conduct of the TGR and the *Pig-a* assays, respectively:

  - Blood is collected for the *Pig-a* assay.

  - Tissue selection for the TGR mutation assay should be guided by route of administration, ADME, and toxicity data.

  - For the TGR mutation assay, multiple tissues should be analyzed for mutant frequency (Lambert et al. 2005; Nohmi et al. 2017; OECD 2022b). The number of tissues analyzed should be sufficient to address the concern; it should not necessarily be limited to one or two tissues (e.g., stomach, duodenum, liver for oral exposures, lung with inhalation exposure, tissues of highest exposure from a distribution study). The strength of the assay in a WoE assessment of the active ingredient and/or its metabolites is increased by analyzing multiple tissues based upon ADME and toxicity data.

  - Verification of systemic and/or bone marrow exposure using a validated bioanalytical method is necessary to support negative findings in the *Pig-a* gene mutation assay (OECD 2022a).

  - Active ingredient exposure should be verified in tissues selected for mutation analysis to support negative findings in the TGR mutation assay (OECD TG 488 (OECD 2022b)).

- An in vivo micronucleus and/or comet assay, which detect clastogenic large-scale genetic damage, is not considered appropriate to address an Ames-positive response and likely

would fail to detect an active ingredient generating small-scale mutation, which is concerning from a cancer risk perspective (Robison et al. 2021).

D. Considerations for Follow-Up Testing of Ames-Positive Active Ingredient Metabolites

The standard battery of genetic toxicity studies with the active ingredient is generally considered adequate to assess the genotoxic potential of the active ingredient (or parent drug) and metabolites.

- An Ames-positive metabolite should be evaluated for in vitro and in vivo mutagenicity in a similar manner as discussed above for the active ingredient. In some circumstances, it may be advantageous to test the isolated metabolite.

- Human disproportionate or novel metabolites are handled as described in Note 4.

- An Ames-positive metabolite observed at low levels (e.g., µg concentrations), regardless of the percentage level relative to total active ingredient, would generally pose minimal safety concerns to healthy human subjects. An approach comparable to that described in ICH M7(R2) for low level genotoxic impurities (e.g., at the approximate threshold of toxicological concern) could be used without extensive follow-up in vitro and in vivo genotoxicity testing as described above.

E. Need for Carcinogenicity Testing

When considered as part of a WoE assessment, negative results in both an in vitro mammalian mutation assay (i.e., the MLA or HPRT assay) and an in vivo mutation assay can potentially contribute to an adequate safety assessment for conducting FIH clinical trials in healthy human subjects. Follow-up testing cannot entirely mitigate the concerns raised by an Ames-positive finding, and some residual risk remains in the absence of an adequate carcinogenicity assessment. Enrollment of healthy subjects in FIH trials should be justified (i.e., why are healthy subjects being administered the investigational new drug containing the active ingredient of interest rather than patients, is adequate information in the Informed Consent document to describe the findings of mutagenic potential so subjects are aware of the potential risk). In most cases, however, rodent carcinogenicity studies would still be expected prior to or with the submission of a new drug application, and such studies, if needed, may be conducted earlier during the IND drug development phase.[18,19]

Carcinogenicity studies could be considered for an Ames-positive active ingredient that was also positive in the in vitro mammalian assay and/or in vivo mutation assay to determine if the

[18] Refer to ICH S1A, ICH S1B, ICH S1B(R1), ICH S1C(R2), and ICH S9.

[19] We support the principles of the "3Rs," to reduce, refine, and replace animal use in testing when feasible. We encourage sponsors to consult with us if they wish to use a non-animal testing method they believe is suitable, adequate, validated, and feasible. We will consider if such an alternative method could be assessed for equivalency to an animal test method.

positive signals translate into tumor findings. A negative carcinogenicity study would be needed to support an FIH trial in healthy subjects under these circumstances.

IV. CONSIDERATIONS FOR CONDUCTING THE WOE ASSESSMENT OF AN AMES-POSITIVE ACTIVE INGREDIENT OR METABOLITE

Recommendations for follow-up testing of an Ames-positive active ingredient or metabolite are presented in the decision tree in Figure 1.

WoE considerations for the safety of conducting FIH trials with an investigational new drug containing an Ames-positive active ingredient, from evaluating the Ames response (decision tree, box 5) to considering the results of in vitro mutagenicity testing (decision tree, boxes 3a, 3b or boxes 8a, 8b) and in vivo mutagenicity testing (decision tree, boxes 10a, 10b), are presented in Figure 1 and discussed below.

- The structure of the active ingredient should be evaluated through use of quantitative structure activity relationship analysis and read across for potential structural alerts indicating mutagenic activity. If feasible, data from closely related structures of other chemicals with known mutagenicity and/or carcinogenicity information can be informative. Information from a different chemical that possesses the same structural alert(s) within a comparable environment and is known to be negative for carcinogenicity could potentially be used to reduce the level of concern for mutagenicity in a WoE evaluation. Alternatively, information from a different chemical that possesses the same structural alert(s) within a comparable chemical environment and is known to be positive for carcinogenicity would increase the level of concern; this could potentially preclude further testing and indicate that administration of the active ingredient to healthy subjects is not acceptable without further justification.

- Rodent and human metabolite profiles should be evaluated (see also discussion of metabolites in section D). An in vitro positive result attributed to a rodent-specific metabolite that is not relevant to humans could be used to reduce or eliminate the concern for mutagenicity. Alternatively, evidence for a potentially mutagenic human metabolite not generated by rodent metabolism might increase the concern. Examining metabolic profiles may also provide evidence of rapid in vivo inactivation of the mutagenic form (e.g., prodrug that forms a non-mutagenic metabolite, metabolism to a non-mutagenic form, breakdown of the mutagenic form in stomach). Such data could be used to reduce the level of concern. Further follow-up testing under these circumstances might be unnecessary with sufficient evidence and supporting justification. Follow-up studies with human S9 could be conducted, although the variability in human S9 preparations limits their value and could only be considered as one factor in an extensive WoE evaluation.

- Any of the following factors alone would indicate that FIH trials in healthy subjects is not recommended:

- The active ingredient has a comparable structure to a known carcinogen. Chemicals in the cohort of concern (e.g., N-nitroso, polyaromatic hydrocarbon, aflatoxin-like, alkyl-azoxy compounds) are excluded from consideration of follow-up testing as described in this guidance. FIH trials in healthy subjects would not be appropriate.

- A positive or equivocal response in the MLA or HPRT test would preclude trials in healthy subjects (see boxes 3a and 8a in the decision tree); no further testing is recommended. Positive responses in additional assays of the standard battery of genetic toxicity tests (e.g., in vitro chromosomal aberration assay, in vivo micronucleus assay) would add further evidence to preclude trials in healthy subjects. The only path forward might be to conduct FIH trials in patients with the disease or condition if there is an acceptable risk-benefit ratio or, alternatively, conduct a 6-month rasH2 mouse or 2-year rat or mouse carcinogenicity study. If such studies are conducted, the results should be negative to continue development and conduct FIH trials in healthy subjects.

- An Ames-positive active ingredient that was negative in the MLA or HPRT test (see decision tree, boxes 3b and 8b) but positive in the TGR mutation assay and/or *Pig-a* assay would indicate positive in vivo mutagenic potential. Therefore, FIH trials in healthy subjects would not be supported (see decision tree, box 10a). The only path forward might be to conduct FIH trials in patients with the disease or condition if there is an acceptable risk-benefit ratio or, alternatively, conduct a 6-month rasH2 mouse or 2-year rat or mouse carcinogenicity study. If such studies are conducted, the results should be negative to continue development and conduct FIH trials in healthy subjects.

- In most cases, based on evaluation of follow-up tests (e.g., positive in either an in vitro MLA or HPRT assay or in vivo mutation assay, and others), it may become apparent that conducting FIH trials in healthy human subjects with an investigational new drug containing an Ames-positive active ingredient is inappropriate and that conducting additional follow-up testing is not recommended.

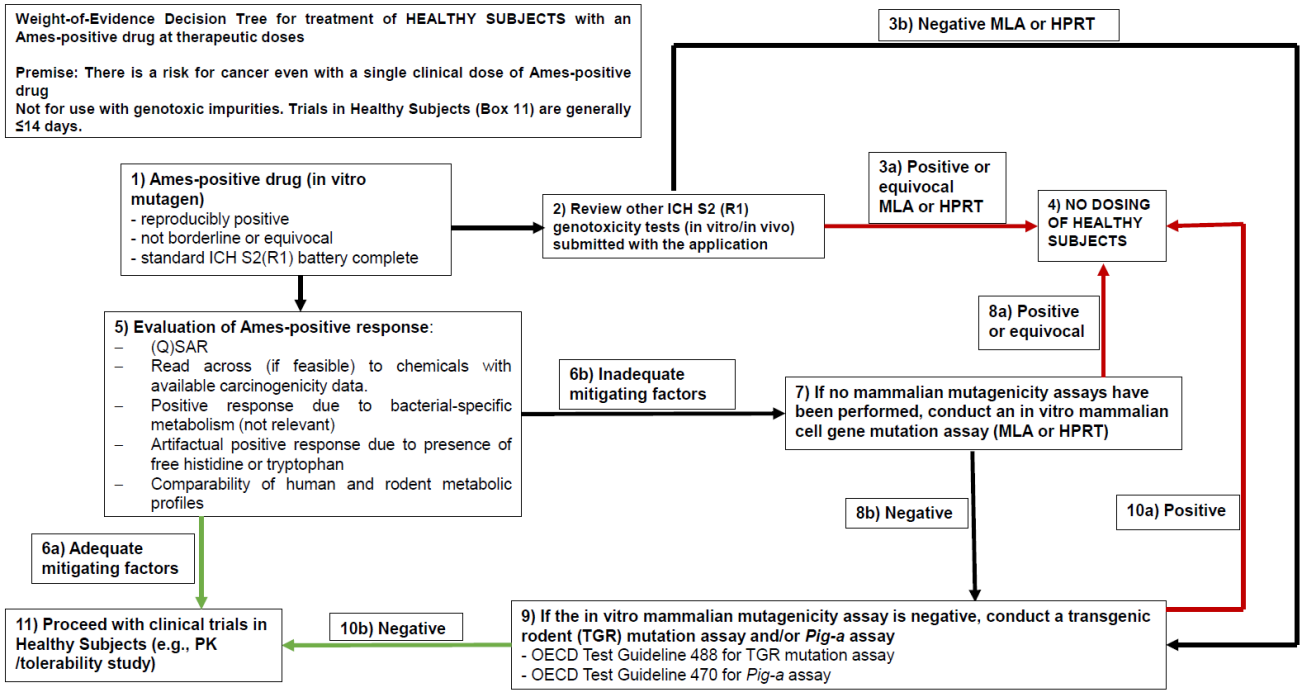
- In those rare occasions when the circumstances, including evidence of safety (i.e., negative in vitro MLA or HPRT test and negative in vivo mutation assay) or mitigating factors, are sufficiently compelling, it may be possible to conclude that it is reasonably safe to proceed with FIH trials in healthy human subjects with an investigational new drug containing an Ames-positive active ingredient (decision tree, box 10b). However, the recommended follow-up tests alone do not fully mitigate a positive Ames test finding, and some residual uncertainty remains. Thus, in the absence of adequate mitigating factors, Ames-positive active ingredients should only be developed for serious or life-threatening diseases with unmet medical needs.

V. RECOMMENDATION TO CONSULT WITH CDER REVIEW DIVISION

Sponsors should seek input through the pre-IND process from the appropriate CDER review division before submission of an IND that proposes FIH trials in healthy subjects that involve administration of an Ames-positive active ingredient.

The figure displays a decision tree for follow-up of an Ames-positive active ingredient (or metabolite) in terms of an evaluation of the response (box 5) and subsequent follow-up testing that includes an in vitro MLA or mammalian cell HPRT assay (box 2 or 7) and an in vivo transgenic rodent mutation assay and/or *Pig-a* assay (box 9). Both the in vitro mammalian cell gene mutation assay and in vivo gene mutation assay would need to be negative for considering a FIH trial with healthy subjects (box 11). If either the in vitro mammalian cell gene mutation assay or the in vivo gene mutation assay was positive, a FIH trial with healthy subjects would not be supported (box 4).

Figure 1: Decision Tree for Follow-Up Testing With an Ames-Positive Drug (Active Ingredient)



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[20] Some of the listed references also apply to the appendix.

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APPENDIX[1]

NOTES:

Note 1. Evidence that single doses or short-term dosing with carcinogenic substances causes cancer

Halmes et al. (2000) found that animals exposed to some carcinogens using a stop-exposure experimental approach could potentially have higher tumor incidences compared with animals that were exposed continuously. Most of the carcinogens in the stop-exposure studies had significantly higher (≥2-fold response) carcinogenic potencies (lower ED_{01}; 1% added cancer risk) than the chronic lifetime exposures for at least one tumor site. Findings from stop-exposure modeling suggest that short-term exposures could pose cancer risks. In general, a long-term or lifetime exposure is necessary for detection of carcinogenic responses; however, this does not preclude that carcinogenic responses also can occur with short-term exposures. Positive responses in the Ames test and/or other genotoxicity tests appeared to be characteristic of chemicals that produced positive cancer results in stop-exposure studies.

Calabrese and Blain (1999) compiled a database of tumor incidences following single exposures to a suspected chemical to estimate risk from less-than-lifetime exposures. The database contained over 5,500 studies for more than 800 chemicals collected from more than 2,000 articles that addressed single-exposure carcinogenesis. Single doses of several chemicals were found to produce tumors in both sexes, in numerous animal models, and for all age groups. Many of these chemicals were members of chemical classes that are known to be potentially positive in the Ames test, including polyaromatic hydrocarbons, nitrosamines, aromatic amines, and azo compounds. Tumorigenic responses were observed with single exposures to chemicals with wide structural diversity and in all principal animal models, implying that humans are likely to exhibit a qualitatively similar response. Positive responses in the Ames test and/or other genotoxicity tests appeared to be a potential commonality in positive cancer responses following a single dose.

Note 2. Evidence of a strong relationship between positive findings in the Ames test and rodent carcinogenicity (RC) testing

From a database (EPA GENE-TOX) of 3,596 chemicals with genetic toxicity data, 1,607 (44.7%) had Ames (*Salmonella*) data and 988 (27%) also had rodent carcinogenicity study data (Table 1, (Matthews et al. 2006)). Table 1 lists the numbers for the chemicals that were evaluated in both the Ames test and an RC study that were true positives (a: both the Ames test and the RC study were positive), false negatives (b: the Ames test was negative, but the RC study was positive), false positive (c: the Ames test was positive, but the RC study was negative), and true negatives (d: both the Ames test and the RC study were negative). The concordance between responses in the Ames test and 2-year RC study was 78.3% (Table 2, (Matthews et al. 2006)). The positive predictivity for Ames-positive chemicals also being positive in the RC study was 76.4%, with the frequency of mutagenic noncarcinogens being less than 20%. Thus, an Ames-positive result is highly predictive of a positive tumorigenic response in the 2-year RC study.

[1] Some of the references are listed in the References section to the guidance.

Table 1. Results With 988 Chemicals With Ames (*Salmonella*) Data and RC Study Data[1]

	Carcinogenic	
Mutagenic (Ames)	**Positive**	**Negative**
Positive	275 **a** (True +)	85 **c** (False +)
Negative	282 **b** (False -)	346 **d** (True -)

[1]Derived from Matthews et al. (2006).

Table 2. Concordance Between Ames (*Salmonella*) Data and RC Study Data[2]

Parameter	Percentage	From Table 1	Classification
Overall concordance	62.9%	a+d/(a+b+c+d)	Fraction of matching results
Sensitivity	49.4%	a/(a+b)	Fraction of carcinogens that are mutagens
Positive predictive value	76.4%	a/(a+c)	Fraction of mutagens that are carcinogens
Specificity	80.3%	d/(c+d)	Fraction of noncarcinogens that are not mutagens
False positives	19.7%	c/(c+d)	Mutagenic noncarcinogens
False negatives	50.6%	b/(a+b)	Non-mutagenic carcinogens
Correlation indicator	78.3%	n/a	Indicator of a positive finding in the RC bioassay

[2]Derived from Matthews et al. (2006).

Cheeseman et al. (1999) evaluated Ames test data for 442 chemicals out of a cohort of 709 carcinogens. Comparisons of the potencies of Ames-positive and Ames-negative carcinogens found that, on average, Ames-positive carcinogens were eight times more potent in terms of tumorigenic dose-response. Mutagenic carcinogens were approximately three times more likely to be potent carcinogens than non-mutagenic carcinogens. Thus, a finding of in vitro mutagenicity raises the concern that the test article may be a potent carcinogen.

Figure A. Tumorigenic Potency of Ames-Positive and Ames-Negative Carcinogens[2]

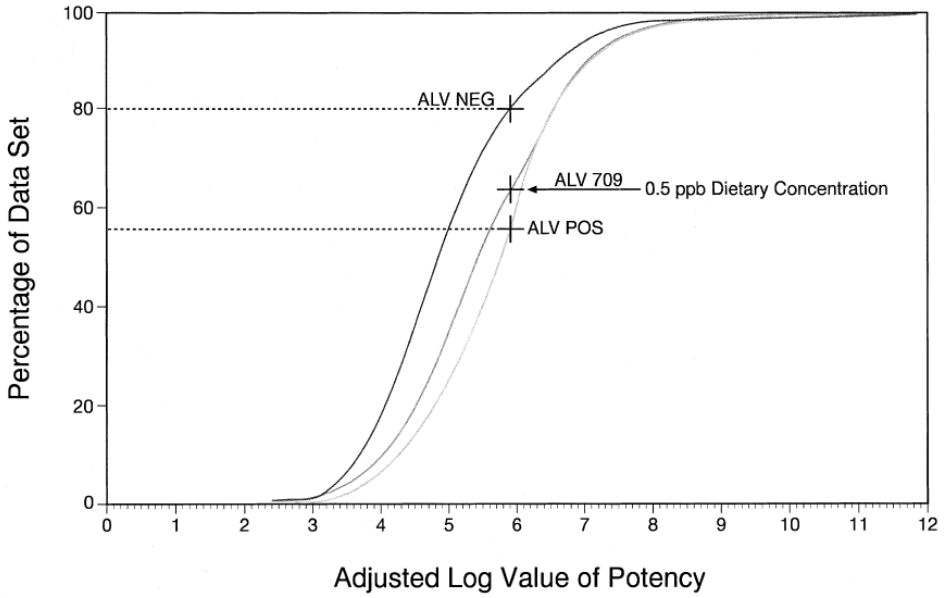

Figure A. Adjusted log value of potency versus percentage of data set.
ALV Neg = adjusted log value of compounds negative in the Ames assay.
ALV 709 = adjusted log value of compounds in the cohort of 709.
ALV POS = adjusted log value of compounds positive in the Ames assay.

Investigations conducted by Halmes et al. (2000) and Calabrese and Blain (1999) indicate cancer can potentially develop with a single dose or short-term exposure to an Ames-positive chemical.

Note 3. Support for use of the in vitro mouse lymphoma assay (MLA) or mammalian cell hypoxanthine-guanine phosphoribosyl transferase (HPRT) test as follow-up for testing Ames-positive substances to identify rodent carcinogens

Kirkland et al. (2014) showed that the combination of an in vitro MLA or mammalian cell HPRT test plus an in vitro mammalian cell assay for another endpoint (e.g., chromosomal aberrations or micronucleus) has been proposed to have a high correlation to Ames-positive in vivo carcinogens. To identify whether an Ames-positive chemical is predicting the in vivo positive response of the chemical, it would be important to know whether the chemical is genotoxic in vitro in mammalian cells (and for what endpoints), whether it has structural alerts (and the type of alerts), and whether data can be obtained from mechanistic in vitro studies that more clearly define the risk. If such data indicate a lower possibility of carcinogenic or in vivo mutagenic potential, it may indicate that in vivo testing can be avoided or minimized.

The incidence of Ames-positive chemicals with negative results in two mammalian cell assays was as follows:

[2] Taken from Cheeseman et al. (1999).

- 1.2% (1/86) for in vivo genotoxic compounds
- 3.3% (9/211) for carcinogens
- 21.4% (15/70) for in vivo non-genotoxic compounds
- 22.6% (12/53) for noncarcinogens.

If an Ames-positive compound shows negative results in well-performed in vitro mammalian cell tests for both gene mutation and chromosomal damage, the compound is unlikely to be an in vivo genotoxin or carcinogen. It is noted that the database used by Kirkland et al. (2014) was relatively small and primarily consisted of potent carcinogens. Further, the structures and/or groups evaluated were limited.

A test for clastogenicity (e.g., chromosomal aberrations), as suggested by Kirkland et al. (2014), is considered unnecessary for assessing the direct relevance of an in vitro Ames-positive active ingredient given the differences in the endpoints (e.g., small-scale mutation for Ames versus large-scale chromosomal damage for the micronucleus or chromosomal aberration assays). Further, for a chemical that is negative in the MLA or HPRT test, further follow-up testing consists of conducting an in vivo mutation study.

Note 4. Human unique or disproportionate metabolites

Human unique or disproportionate metabolites are unlikely to be known before a first-in-human trial commences as they are typically identified later in development (e.g., human mass balance study). However, the principles for characterization of an Ames-positive unique or disproportionate metabolite, if known, are similar to those for an Ames-positive active ingredient. An Ames-positive unique or disproportionate metabolite is not formed by standard nonclinical test species or only formed at low levels (e.g., the area under the curve in humans is much greater than in nonclinical test species). Also, it is not expected to be formed in vitro by exogenous rat S9 metabolites.[3] In most cases, it will be necessary to synthesize the metabolite for in vitro and in vivo nonclinical studies.

Given the high correlation between an Ames-positive response and a positive tumorigenic response in the 2-year RC study,[4] an Ames-positive metabolite would be handled on a case-by-case basis irrespective of its percentage of total systemic drug exposure. In general, follow-up testing of Ames-positive metabolites, present at less than 10% of total systemic exposure, should be conducted based upon the high level of safety concern; see the potential exception below.[5] A major Ames-positive metabolite (>10%) should more than likely be handled in a comparable manner to the active ingredient, as indicated in ICH M3(R2), the guidance for industry *Safety*

[3] Refer to the ICH guidance for industry *M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals* (January 2010) and the guidance for industry *Safety Testing of Drug Metabolites* (March 2020). We update guidances periodically. For the most recent version of a guidance, check the FDA guidance web page at https://www.fda.gov/regulatory-information/search-fda-guidance-documents.

[4] See ICH M3(R2), Robison et al. 2021, the guidance for industry *Safety Testing of Drug Metabolites*, and Note 2.

[5] See ICH M3(R2), Robison et al. 2021, and the guidance for industry *Safety Testing of Drug Metabolites.*

Testing of Drug Metabolites, and Robison et al. (2021). If further follow-up testing is necessary, studies with the isolated metabolite in the transgenic rodent and/or *Pig-a* gene mutation assay should be considered.

REFERENCES FOR THE APPENDIX

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