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Letter

In vitro genotoxicity test package of antibiotics for human use submitted to the Japanese regulatory agency during 2004−2015

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ABSTRACT — The Ames test is used for the mutagenic assessment of drugs; however, it may not provide an accurate genotoxic profile for bactericidal compounds. This study was performed to clarify 1) whether the total number of genotoxicity assays performed (*#Assays*) was greater during antibiotic development than during the development of other drugs, particularly antivirals, possibly due to the requirement for additional assessments, 2) whether the maximum doses of the Ames test were less when an alternative assay had been performed for antibiotics, and 3) whether some particular alternative assay had an advantage to minimize *#Assays* in the last decade. Genotoxicity data submitted to the Pharmaceuticals and Medical Devices Agency in Japan during 2004−2015 were used. The *#Assays* was greater and the maximum doses of the Ames tests were lower for antibiotics, which was more obvious when alternative mutagenic assays had been performed. The mouse lymphoma assay or hypoxanthine-guanine phosphoribosyl transferase gene mutation assay was performed preferentially as an alternative. For antibiotic development, preferred genotoxicity test packages should be discussed in the future for a better understanding of the genotoxic potential of antibiotics.

Key words: Ames test, Mutagenicity, Mouse lymphoma assay, MLA, Hypoxanthine-guanine phosphoribosyl transferase gene mutation assay, *HgPRT*

INTRODUCTION

When developing pharmaceuticals, such as small molecule compounds, their genotoxicity, especially mutagenicity, is one of the most important safety issues to be assessed before they are used in a first-in-human clinical trial. The Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (ICH S2(R1)) (ICH guidance, S2(R1), 2011) indicates that a reverse mutation assay with bacteria, which is Ames test (Ames *et al.*, 1972) can be used to test the potential mutagenicity of candidate compounds. ICH S2(R1) also indicates that the maximum dose of the Ames test should be 5000 μg/plate or maximum soluble dose, and mutagenic evaluation can be performed up to the dose that causes cytotoxicity. However, since the Ames test uses bacteria, certain chemicals, such as antibiotics targeting Gram-negative bacteria, kill them at a very low dose compared to the 5000 μg/plate. Thus, in such a case, another ICH guidance, Guidance on Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (ICH

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M3(R2)) (ICH guidance, M3(R2), 2009), describes that "an appropriate alternative assay if Ames is inappropriate, for example, for an antibacterial product" should be performed for genotoxicity assessment, indicating that mutagenicity cannot be adequately, appropriately or scientifically evaluated if the dose was not high enough. At the same time ICH S2(R1) says that the Ames test should still be performed and thus "Option 1" should be followed for total genotoxic evaluation, meaning that a genotoxicity test package consists of, at least, the Ames test, an *in vitro* assay using mammalian cells, and one *in vivo* assay. In this context, the *in vitro* assay using mammalian cells has to be capable of assessing both mutagenicity and chromosome abnormality, otherwise assessing each separately. Therefore, the former option can possibly be a mouse lymphoma assay (MLA), and the latter option consists of two separate *in vitro* assays, for example, hypoxanthine-guanine phosphoribosyl transferase (*HgPRT*) gene mutation assay for mutagenicity (OECD GUIDELINE FOR THE TESTING OF CHEMICALS, 2016) and *in vitro* metaphase chromosome aberration test for clastogenicity.

Thus, we decided to analyze data submitted to the Japanese regulatory agency from 2004 to clarify whether the number of genotoxicity assays performed (*#Assays*) has been greater and maximum doses of the Ames test can be less for the development of antibiotics in comparison with the case of antivirals whose pharmacological targets are non-mammalian molecules like antibiotics, and whether a certain alternative *in vitro* mutagenic assay using mammalian cells was preferably selected. From these analyses we could learn mutagenic assessments in antibiotics are still not straightforward even in the last decade.

MATERIALS AND METHODS

We used data from clinical trial notifications, consultations, and new drug applications submitted/provided to the regulatory agency in Japan, that is, the Pharmaceuticals and Medical Devices Agency (PMDA), between 2004 and 2015. Although some submissions were for new routing drugs or combination drugs including already approved component(s), these were included in the study as long as a list of genotoxicity assays conducted and their data were available. The following data analyses were performed: for both antibiotics and antivirals, how many genotoxicity assays were performed for each compound and what were the maximum doses of the Ames test (frameshift type whose maximum doses were similar to those in base substitution type) used to make a mutagenic assessment possible; and for only antibiotics, how many compounds had additionally examined by other *in vitro* mutagenic assay(s) such as the MLA, *HgPRT* gene mutation assay, etc. Data are expressed as the mean ± standard deviation. Differences were considered statistically significant at a *p*-value of less than 0.05 using Student's *t*-test, unless otherwise stated.

RESULTS AND DISCUSSION

Data from a total of 67 antibiotics and 51 antivirals were obtained in this study, but dosing data were available for 59 antibiotics and 46 antivirals. The average *#Assays* was 4.76 ± 1.45 for antibiotics (n = 67) and 3.49 ± 0.97 for antivirals (n = 51), and there was a statistically significant difference between these two drug groups ($p < 0.001$). The maximum doses of the Ames test were significantly greater for the antivirals (4581 ± 1903 µg/plate; n = 46) than the antibiotics (671 ± 1452 µg/plate; n = 59) ($p < 0.001$), indicating that the mutagenicity of most of the antivirals had been well-evaluated by the Ames test itself. Fifteen antivirals had been tested by the MLA, but the main purpose of performing this assay was for assessing clastogenicity (chromosome abnormality) since mutagenicity already had been evaluated by the Ames test and the result of the MLA was negative in all cases. The mutagenicity of 13 out of 59 antibiotics had not been assessed by any *in vitro* test other than the Ames test, and the maximum doses of the Ames tests for these drugs were greater, but the *#Assays* was significantly less, compared to the remaining antibiotics (n = 46) (Fig. 1). The mutagenicity of these 46 antibiotics had been assessed by either or both *in vitro* assay(s) and/or *in vivo* assay(s), in which the MLA was at most cases (n = 29), the *HgPRT* assay without performing the MLA was the second (n = 10) and other assays were for remaining antibiotics (n = 7). Tweleve out of 29 MLA resutls (41.4%) showed negative (MLA-negative) and 17/29 (58.6%) showed positive (MLA-positive) with an increase in both large and small sized colonies, failed to distinguish the result from either mutagenicity or clastogenicity (Lloyd and Kidd, 2012). The second most performed *in vitro* mutagenic assay without performing the MLA was the *HgPRT* assay (n = 10) including one xanthine guanine phosphoribosyl transferase gene mutation assay: 9/10 results showed negative and 1/10 results showed positive. In fact, 6 MLA-positive antibiotics had been additionally assessed by the *HgPRT* assay in order to negate mutagenic potential, which has been successful eventually. Thus, 15/16 antibiotics had given negative results in this assay (maximum concentration: 1588 ± 1614 µg/mL), while caution needs to be taken as the data used in this manuscript are from antibiotics submit-

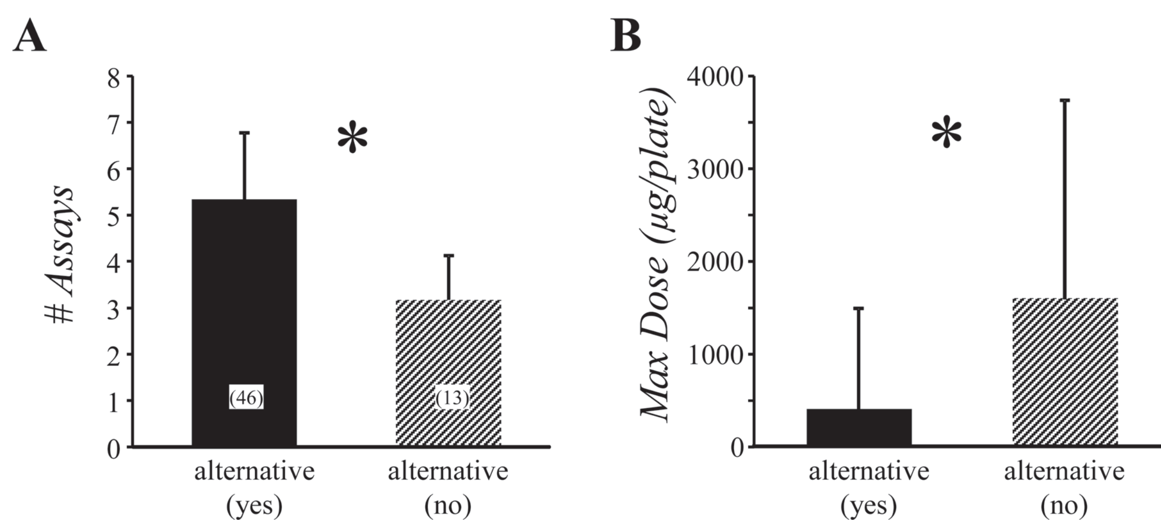

Fig. 1. Comparisons between cases when an alternative mutagenic assay was performed for antibiotics (alternative [yes]) and not performed (alternative [no]) in addition to the Ames test. **A**, total number of genotoxicity assays performed (*#Assays*). The difference was statistically significant (*$p < 0.001$). **B**, maximum dose of the Ames test (frameshift mutations). The difference was statistically significant (*$p = 0.037$). The number in parentheses is the number of antibiotics.

ted to the PMDA as a result of no concern for the genotoxic risk. The mutagenicity of the 7 remaining antibiotics as well as 11 MLA-positive and 1 *HgPRT*-positive antibiotics described above, had been evaluated by other mutagenic *in vitro* assay using mammalian cells (n = 3) or non-mammalian cells (n = 2), or some *in vivo* assay (n = 4), or denied their mutagenic potential based on mechanistic analysis and/or other data (n = 3), otherwise their development was suspended for some reason (not necessarily due to possible genotoxicity risk).

In case that MLA had been selected for alternative mutagenic assessment, the actual average of the *#Assays* was 5.17 ± 1.20 (n = 29), which was not significantly less than for the cases where the MLA had never been selected (5.59 ± 1.42; n = 17) ($p = 0.318$), probably due to the presence of 17 MLA-positive cases. In fact, the *#Assays* for the MLA-negative cases was 4.33 ± 0.98 (n = 12), which was significantly less than the MLA-positive cases (5.76 ± 0.97; n = 17) ($p < 0.001$). In addition, the maximum doses of the Ames tests for the MLA-negative cases was 1330 ± 1862 μg/plate (n = 12), which was significantly greater than the MLA-positive cases (133 ± 250 μg/plate; n = 17) ($p = 0.014$), while the maximum concentration of the MLA did not show any differences between their assay results. In fact, the maximum dose of the Ames test and the maximum concentration of the MLA do not look like co-related (Fig. 2), indicating that cytotoxic mechanism(s) is different between bacteria and mammalian cells. Though it is not clear why MLA-positive antibiotics showed a tendency to have a lower maximum dose in the Ames test which situation means potential mutagen has not been evaluated, it may be worth to try MLA as screening for antibiotics to pick up the potential mutagen.

This study re-confirms that more genotoxicity tests were required during the development of antibiotics because of the low level of maximum dose of Ames test, which would be considered that the mutagenicity assessment is not enough. The most common alternative *in vitro* mutagenic assay using mammalian cells was the MLA, which may or may not be responsible for causing problematic mutagenicity assessments frequently seen in this study. The MLA, which is exampled in the ICH S2(R1) guideline and can evaluate both mutagenicity and chromosome abnormality, should possibly and would preferably be selected as an alternative *in vitro* assay as confirmed. Some of the MLA-positive cases were from positive results of high concentration (> 500 μg/mL) of test solution and possibly due to the high sensitivity of the assay (Morita *et al.*, 2014; Parry *et al.*, 2010), while these positive results had eventually been negated by other assays. Thus, the new assays, for example, *in vitro* PIG-A gene mutation assay (Rees *et al.*, 2017), may be needed for better understanding mutagenicity of antibiotics as well as other pharmaceuticals.

A limitation of this study comes from the fact that we

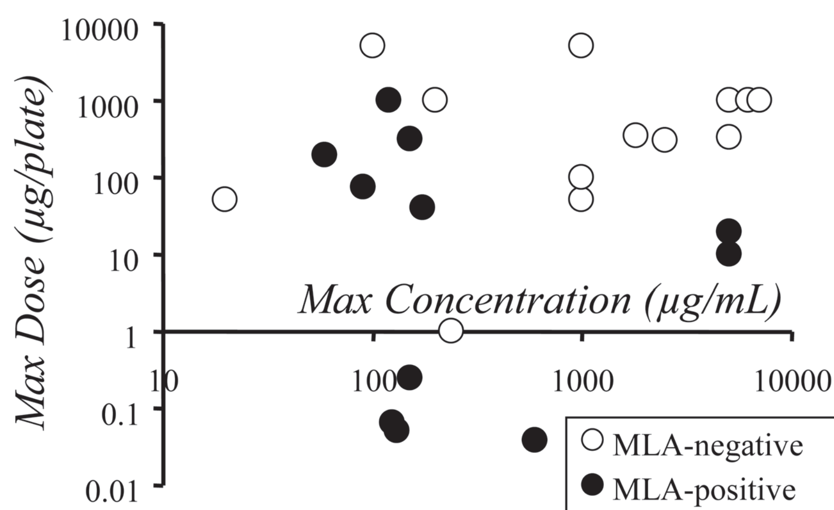

Fig. 2. Scatter plots of the maximum dose of the Ames test against the maximum concentration of the MLA. Both x-axis and y-axis have a logarithmic scale. Those two parameters do not look like co-related, however plots from MLA-negative data seems to be distributing relatively upper side of the graph.

only analyzed data that were submitted to the PMDA, and it could be possible that more chemicals (antibiotics) might have shown positive results in both the MLA and the *HgPRT* assay and had never been submitted to the agency. Thus, probably, each individual drug developer might have a different strategy, such as choosing or not choosing the MLA initially, based on their own previous experiences of antibiotic development. Nevertheless, our results may suggest that someone may clearly refer to the limitation of choosing the MLA and select the other option of genotoxicity test packages for antibiotics, especially those targeting Gram-negative bacteria.

As shown in this study, the maximum dose cannot be 5000 μg/plate in the Ames test especially for antibiotics, how high should the dose be to adequately assess the mutagenicity; 10, 100 or 1000 μg/plate? There is a report showing that strong mutagens can be detected by the Ames test in which the maximum dose required is only up to 250 μg/plate (Kenyon *et al.*, 2007). However, even not-so-strong mutagenic compounds may potentially have a greater risk, when significant amounts of them are intentionally and/or frequently taken in practice, such as in the case of antibiotics (e.g. 6.75 g piperacillin can be injected intravenously). We know that all assays have limitations; however, in future, it will be better to have an indication of how reasonably true the Ames test results are at less than 5000 μg/plate of the maximum dose. We will have to wait for further scientific progress and discussions by experts to resolve this issue.

Given the limited number of available data, the pharmacological and toxicological properties of each individual antibiotic could be quite different. In this study, there were 5 quinolones all of which had shown positive results in the MLA. It is known that quinolones inhibit DNA synthesis in bacteria via DNA gyrase, and also inhibit eukaryotic topoisomerase II, which is homologous to DNA gyrase in mammals. Thus, it is considered that quinolones causes chromosomal aberrations via double strand breaks during DNA synthesis through topoisomerase II inhibition (Morita *et al.*, 2014; Parry *et al.*, 2010). This might be in agreement with the positive results of the MLA, but that mechanism may not be the only one. On the contrary, the *HgPRT* assay was performed in 7 quinolones including 2 MLA-positive ones, and only one quinolone showed positive result. Interestingly, chemical-specific point mutational profiles in the *HgPRT* assay were considered to be similar to those in the MLA (Chen *et al.*, 2002), while positive results can definitely be found in the MLA assay more than the *HgPRT* assay because of the characteristics of the MLA (Johnson *et al.*, 2012).

In conclusion, this study showed that mutagenicity has not been assessed adequately by the Ames test for most of the antibiotics analyzed because of strong pharmacological (bactericidal) effects confirmed by the low level of the maximum dose in the test. Alternatively, other *in vitro* mutagenic assays needed to be performed, the majority of which was the MLA, as recommended in Option 1 of ICH S2(R1). Although this study could not really

suggest a gold standard of genotoxicity testing packages for any antibiotic, mutagenic assessments of antibiotics seems to be struggled even in the last decade and thus more straightforward genotoxicity risk assessments might be required in the future.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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