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Minireview

A survey of on the use of genotoxicity and carcinogenicity testing packages for ophthalmic drug development

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ABSTRACT — For the non-clinical safety evaluation of pharmaceuticals for new drug applications (NDA), various toxicity studies must be conducted at each stage, from clinical trials to NDA. For topically applied drugs, the level of exposure at the administration site is high because the drug is administered directly to the administration site. However, because systemic exposure to ophthalmic drugs is generally lower than that of systemic drugs, systemic effects may not be adequately assessed. The bone marrow and liver are generally evaluated after systemic administration in *in vivo* genotoxicity tests, and local genotoxicity studies are conducted on a case-by-case basis. Therefore, we surveyed packages of genotoxicity and carcinogenicity tests for ophthalmic drugs approved in Japan from 2004 to 2021 to assist in the decision of test packages for the development of ophthalmic drugs. There were no major differences in genotoxicity test packages compared to systemic drugs; however, an unscheduled DNA synthesis test using the cornea after ocular instillation was conducted in some products as a test specific to ophthalmic drugs. In the development of ophthalmic drugs, if a positive result is found in an *in vitro* genotoxicity test, the safety margin between the positive concentration and the clinically applicable concentration (eye drop concentration) is required for safety assessment. If the safety margin cannot be ensured, additional tests may support safety assessment.

Key words: Ophthalmic drug development, Genotoxicity test, Carcinogenicity test, Study package

INTRODUCTION

Non-clinical safety evaluation for the new drug application (NDA) of pharmaceuticals usually comprises acute toxicity, repeated-dose toxicity, reproductive and developmental toxicity, genotoxicity, carcinogenicity, immunotoxicology, photosafety, and other toxicity studies. The International Conference on Harmonisation (ICH) guidance on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals (ICH M3 guidance) states that these non-clinical studies should be conducted at each stage from clinical trials to NDA (ICH, 2009). During their development, topically applied drugs are administered directly to the application site, such as the skin for transdermal creams and the cornea for eye drops. However, systemic toxic effects may not be adequately assessed for topical administration, such as ocular instillation, because systemic exposure is less than that for oral or intravenous administration. Therefore, repeated-dose toxicity studies after systemic administration should be conducted along with clinical-route repeated-dose toxicity studies to develop topically applied drugs. Nevertheless, *in vivo* genotoxicity is routinely performed on bone marrow and liver after systemic administration, whereas genotoxicity studies with topical administration are performed on

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a case-by-case basis. Therefore, we surveyed the genotoxicity and carcinogenicity testing packages for ophthalmic drugs approved by the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan over 18 years period from 2004 to 2021 to contribute to the decision of these study packages in the development of ophthalmic drugs.

Prescription drugs include new and generic drugs; new drugs are mainly classified into six categories: drugs with a new active ingredient, new combination drugs, drugs with a new administration route, drugs with a new indication, drugs with a new dosage, and drugs in a new dosage form. Many ophthalmic drugs with a new active ingredients, new administration routes, and new combinations have been developed. All toxicity studies required for applying a new ophthalmic drug with a new active ingredient must be conducted. For drugs with a new administration route, numerous toxicity tests have been conducted during the development of systemic drugs. When an eye drop is developed in the new administration route category, many toxicity tests are omitted by citing the original drug's common technical document (CTD). For new combination drugs comprising multiple active ingredients, additional toxicity studies must be considered based on the safety profile of the individual ingredients and the degree of experience with concomitant administration in clinical use. Additional studies are required for drugs with a new indication, new dosage, and new dosage form depending on the degree of change. We focused on two of these categories: drugs with a new active ingredient and a new administration route and surveyed and discussed the differences in genotoxicity and carcinogenicity testing packages.

In addition, many *in vivo* genotoxicity studies after topical administration have been reported, including the comet assay, micronucleus assay, and unscheduled DNA synthesis (UDS) test using mouse skin after percutaneous administration (Haesen *et al.*, 1993; Toyoizumi *et al.*, 2011). In the development of CORECTIM® ointment 0.5%, approved in 2020, the micronucleus test following percutaneous administration was conducted as an *in vivo* genotoxicity test (PMDA, 2020). However, few genotoxicity studies have been conducted on the ocular surface after ocular instillation, which is often an issue in eye drop development. Therefore, we have summarized the literature on genotoxicity tests with ocular tissues and discussed useful evaluation systems for developing ophthalmic drugs.

In this review, genotoxicity and carcinogenicity testing packages for ophthalmic solutions and intravitreal injections approved as new drugs in Japan were surveyed with reference to CTDs, and differences in test packages by application classification were discussed. In addition, genotoxicity tests with ocular tissues are summarized, and their usefulness is discussed in the development of ophthalmic drugs.

MATERIALS AND METHODS

We used the CTDs for ophthalmic drugs approved as new drugs in Japan over 18 years from 2004 to 2021 and published papers on genotoxicity tests with ocular tissues.

RESULTS

Genotoxicity tests and carcinogenicity tests for eye drop development

Table 1 shows the number of eye drops approved as new drugs in Japan between 2004 and 2021. Of the thirty approved drugs, eight had a new active ingredient, nine had a new administration route, and eight were new combination drugs. In addition, drugs with new indications and new dosages were approved.

Drugs with new active ingredients

A summary of the genotoxicity and carcinogenicity test results of the eight drugs with new active ingredients is shown in Table 2. The most common genotoxicity tests were a combination of the Ames test, *in vitro* chromosome aberration test, mouse lymphoma thymidine kinase (tk) test, and *in vivo* micronucleus test. Five products showed positive or pseudo-positive results in the *in vitro* genotoxicity tests.

Table 1. The number of ophthalmic drugs approved from 2004 to 2021 in Japan.

	Total	New active ingredient	New route	New combination	Others	Biosimilar
Eye drops	30	8	9	8	5	−
IVT	7	5	1	−	−	1

IVT, intravitreal injection drugs; New active ingredients, drugs with a new active ingredient; New route, drugs with a new administration route; New combination, new combination drugs.

Table 2. Genotoxicity and carcinogenicity testing packages for eye drops approved as drugs with a new active ingredient in Japan.

	Approval	Ames test	*In vitro* tests			*In vivo* tests			Carcinogenicity tests	References
			CA	MLA	Transformation test	MN	CA	Corneal UDS		
TRAVATANZ® ophthalmic solution 0.004%	2007/7/31	Negative		Pseudo-positive (test 1), negative (test 2)	Pseudo-positive	Negative Mice, i.v., 1 day	Negative Rat, i.v., 1 day		Negative Mice, s.c., 2 years Rat, s.c., 2 years	PMDA, 2007
TAPROS® ophthalmic solution 0.0015%	2008/10/16	Negative	Negative			Negative Mice, i.p., 1 day			Negative Mice, s.c., 1.5 years Rat, s.c., 2 years	PMDA, 2008b
LUMIGAN® ophthalmic solution 0.03%	2009/7/7	Negative		Negative		Negative Mice, i.v., 1 day			Negative Mice, p.o., 2 years Rat, p.o., 2 years	PMDA, 2009
DIQUAS® ophthalmic solution 3%	2010/4/16	Negative	Negative	Negative		Negative Mice, i.p., 1 day				PMDA, 2010b
NEVANAC® ophthalmic suspension 0.1%	2010/10/27	Negative	Positive	Negative		Negative Mice, p.o., 1 day				PMDA, 2010a
AIPHAGAN® ophthalmic solution 0.1%	2012/1/18	Positive	Negative					Negative Rabbit, ocular instillation, 1 day	Negative Mice, p.o. (diet), 91 weeks Rat, p.o. (diet), 2 years	PMDA, 2012a
GLANATEC® ophthalmic solution 0.4%	2014/9/26	Negative	Positive			Negative Rat, p.o., 2 days		Negative Rabbit, ocular instillation, 1 day		PMDA, 2014a
EYBELIS® ophthalmic solution 0.002%	2018/9/21	Negative		Positive		Negative Rat, s.c., 2 days				PMDA, 2018

CA, chromosome aberration test; MLA, mouse lymphoma tk test; MN, micronucleus test; UDS, unscheduled DNA synthesis test.

In the TRAVATANZ® ophthalmic solution (0.004%) approved in 2007, pseudo-positive results were observed in the mouse lymphoma tk and transformation tests. However, the negative results of other genotoxicity tests, such as the Ames test, *in vivo* micronucleus test, *in vivo* chromosome aberration test, and carcinogenicity test, suggested low genotoxic potential. In addition, the concentration at which *in vitro* genotoxicity test showed pseudo-positive results was 800,000 times higher than the systemic exposure (C_{max}) in clinical trials, suggesting a sufficiently high safety margin for clinical use (PMDA, 2007).

The NEVANAC® ophthalmic suspension (0.1%), approved in 2010, showed structural and numerical abnormalities in the *in vitro* chromosome aberration test. However, the positive concentration in the *in vitro* chromosome aberration test was more than 1.5 million times higher than the systemic exposure in clinical trials. In addition, negative results were observed in other genotoxicity studies, such as the Ames test, mouse lymphoma tk test, and *in vivo* micronucleus test. Therefore, the overall risk of inducing genotoxicity was low (PMDA, 2010a).

For the AIPHAGAN® ophthalmic solution (0.1%), approved in 2012, an increase in the number of reverse mutation colonies was observed in the TA1537 strain of the Ames test. In addition, the concentration that yielded positive results in the Ames test was close to the clinical dose (eye drop concentration). However, the reverse mutation potential was relatively weak because there were slight increases of 2–6 times compared to the negative control in the Ames test. Moreover, systemic carcinogenicity tests were negative. To assess genotoxic concerns in ocular tissues, a UDS test was conducted on rabbit corneas after ocular instillation, which yielded negative results. These results suggested no genotoxic concern for ocular tissues in clinical use (PMDA, 2012a, 2012b).

In the GLANATEC® ophthalmic solution (0.4%), approved in 2014, *in vitro* chromosomal aberration test showed an increase in polyploid cells, which was due to pharmacological changes. The concentration at which the number of polyploid cells did not increase in the *in vitro* chromosomal aberration test was 14,000 times higher than that of systemic exposure in clinical trials. In addition, the *in vivo* micronucleus test was negative at approximately 8,200 times the C_{max} of the clinical trials, and the corneal UDS test was negative at five times the clinical dose (eye drop concentration), indicating that there is no concern about genotoxicity occurrence *in vivo*. Moreover, no abnormalities, such as increased multinucleated cells, were observed on the ocular surface in repeated ocular toxicity studies. These results suggest that increases in polyploid cells are unlikely to occur during clinical use (PMDA, 2014a, 2014b).

For the EYBELIS® ophthalmic solution (0.002%), approved in 2018, an increase in small colonies was observed in the mouse lymphoma tk test. However, chromosome-level damage, such as increases in the frequency of small colonies, is generally considered to have a threshold for genotoxicity. In addition, the concentration at which colonies did not increase was 20 times higher than that of the clinical dose (eye drop concentration), and it may not accumulate in the ocular tissues. The results suggested no genotoxic concern in ocular tissue during clinical use (PMDA, 2018).

No carcinogenicity tests were conducted on half of the products surveyed. The reasons included low systemic exposure, low tissue accumulation, no *in vivo* genotoxic concern, no structure–activity relationships suggesting carcinogenic concerns, and no neoplastic or preneoplastic lesions observed in the non-clinical toxicity studies. For the EYBELIS® ophthalmic solution (0.002%), the carcinogenic potential was considered in terms of the threshold of toxicological concern (TTC), which is the tolerable upper intake level of mutagenic impurities in the ICH M7(R1) guideline (ICH, 2017). In other words, the daily dose at the applied concentration was less than TTC (1.5 μg/person/day). In addition, pharmacokinetic studies did not reveal any evidence of blood or organ-specific accumulation. Therefore, a carcinogenicity test was not conducted during the development of the EYBELIS® ophthalmic solution (PMDA, 2018). Carcinogenicity tests were conducted on the four products, all of which used rats and mice. The administration route was oral or subcutaneous, with a dosing period of 2 years for most products.

Drugs with new administration routes

For drugs with new administration routes, genotoxicity studies were conducted during the development of oral or injectable drugs, mainly a combination of the Ames test, *in vitro* chromosome aberration test, and *in vivo* micronucleus test. In addition, *in vitro* studies included forward mutation assays or sister chromatid exchange studies, and *in vivo* studies included chromosome aberration tests or dominant lethal studies. Additional genotoxicity tests were conducted for only two products used for developing eye drops. For the MUCOSTA® ophthalmic suspension UD (2%), approved in 2011, although the Ames test, DNA repair test, *in vivo* micronucleus test, and carcinogenicity test yielded negative results, increased structural aberrations under metabolic activation conditions were observed in the *in vitro* chromosome aberration test

during the oral drug development. Therefore, a forward mutation assay using mouse lymphoma cells and a UDS test using rabbit corneas were conducted during ophthalmic solution development. All additional genotoxicity tests yielded negative results, and there were no concerns regarding genotoxicity in other non-clinical tests. The metabolite level was below the detection limit after a single ocular instillation in rabbits, suggesting that the metabolite is unlikely to be produced in ocular tissues in clinical use. For these reasons, the genotoxic potential of ocular tissues in clinical use is judged to be extremely low (PMDA, 2011). For ALESION® ophthalmic solution (0.05%), approved in 2013, although the transformation assay, *in vivo* micronucleus test, *in vivo* UDS test, and carcinogenicity test yielded negative results, weak positive reactions were observed in the Ames test with the TA1538 strain, which is not a recommended strain in the guidelines and *in vitro* chromosome aberration test during the development of the oral drug. Therefore, the Ames test and *in vitro* chromosome aberration test were conducted using the drug substance following optimization of the manufacturing process, both of which showed negative results. In addition, the UDS test using rabbit cornea was conducted to evaluate genotoxicity in ocular tissues, and no genotoxicity was observed on the ocular surfaces at the clinical dose (eye drop concentration). No neoplastic lesions were observed on the ocular tissue in repeated ocular instillation toxicity studies. Moreover, the concentration yielding negative results in the *in vitro* chromosome aberration test was more than 100 times higher than the estimated ocular tissue concentrations in clinical use. These findings suggest that the genotoxic potential for ocular tissues in clinical use is extremely low (PMDA, 2013).

No additional carcinogenicity studies have been conducted for developing eye drops because most products have already been performed during the development of oral or injectable drugs.

Genotoxicity test and carcinogenicity test for the development of intravitreal injection drug

For intravitreal injection drugs, seven new drugs were approved in Japan from 2004 to 2021, of which five were with new active ingredients, and the others were drug with a new administration route and a biosimilar (Table 1). Four of the five drugs with new active ingredients were biopharmaceuticals; therefore, genotoxicity tests were not conducted according to the ICH S6(R1) guideline (ICH, 2011). For MACUGEN® IVT Inj. KIT (0.3 mg) approved in 2008, the Ames, mouse lymphoma tk, transformation, and *in vivo* micronucleus tests were conducted as genotoxicity tests. No carcinogenicity tests were conducted because the drug is administered intermittently and exhibits antitumor activity as a pharmacological effect. No neoplastic or preneoplastic effects were observed in chronic intravitreal administration studies (PMDA, 2008a).

Summary of genotoxicity tests with ocular tissues

In vivo genotoxicity tests of ocular tissues are summarized in Table 3. In 1987, Nuss *et al.* reported that genotoxicity could be detected using the UDS test with corneal epithelia after UV irradiation in the eyes of rabbits (Nuss *et al.*, 1987), suggesting that genotoxicity is induced in the cornea. Photo-genotoxic agents were orally administered to rats and irradiated with UV light, and then the genotoxicity of the cornea and retina was detected by performing a comet assay (Struwe *et al.*, 2008, 2009). Tahara *et al.* detected genotoxicity by performing the UDS test with corneal epithelia following the ocular instillation of genotoxic agents (Tahara *et al.*, 2021a). They further reported that genotoxicity is detected by performing a comet assay with corneal epithelial cells after the ocular instillation of genotoxic agents (Tahara *et al.*, 2021b, 2022).

For the evaluation of the retina, in addition to the report by Struwe *et al.* described above, de Paula *et al.* reported genotoxicity evaluation in rabbits by performing the comet assay with the retina after intravitreal administration (de Paula *et al.*, 2015).

DISCUSSION

A survey of the genotoxicity testing packages for ophthalmic drugs approved as new drugs in Japan over 18 years period from 2004 to 2021 indicated that the PMDA began to focus on genotoxicity in ocular tissues in 2010 or later. The survey revealed that genotoxicity tests were often a combination of Ames, *in vitro* chromosomal aberration or mouse lymphoma tk, and *in vivo* micronucleus tests during the development of ophthalmic drugs with new active ingredients. The combination of these genotoxicity tests did not differ significantly from that of the test package in the drug development with other administration routes, such as oral or injectable drugs. Positive or pseudo-positive results were observed in the *in vitro* genotoxicity tests for five of the eight approved drugs with new active ingredients. For TRAVATANZ® ophthalmic solution (0.004%), NEVANAC® ophthalmic suspension (0.1%), and GLANATEC® ophthalmic solution (0.4%), there was no genotoxic concern because the concentrations that were negative in the *in*

Table 3. Summary of *in vivo* genotoxicity tests using ocular tissues.

Animals	Treatments	Sampling times	Sampling tissue	Substances	Assay	References
Dutch pigmented rabbits	UV irradiation	Immediately after irradiation	Cornea epithelia	193 nm UV: 400 mJ/cm^2, 248 nm UV: 400 mJ/cm^2, 254 nm UV: 0.5–1.0 J/cm^2	UDS test	Nuss *et al.*, 1987
Kunming mice	Single oral administration	24 hr after administration	Retina	Chlorpyrifos	Comet assay	Yu *et al.*, 2008
Wistar rats	7 J/cm^2 UVA irradiation (24 min) after single oral administration	Immediately after irradiation	Cornea, retina, skin	Sparfloxacin, dacarbazine, chloropromazine HCl, promzine HCl, 8-methoxypsoralen	Comet assay	Struwe *et al.*, 2008
Wistar rats	7 J/cm^2 UVA irradiation (25 min) after single oral administration	Immediately, 1, 2, 3, or 6 hr after irradiation	Cornea, retina, skin	Sparfloxacin	Comet assay	Struwe *et al.*, 2009
New Zealand albino rabbits	Intravitreal administration twice at 30-day intervals	30 d after last administration	Retina	Adalimumab	Comet assay	de Paula *et al.*, 2015
Chinchilla rabbits	H_2S gas exposure (1 hr)	Immediately after exposure	Cornea	H_2S gas	Comet assay	Ibrahim *et al.*, 2018
Japanese white rabbits	Single ocular instillation P.C.: UV irradiation	2 hr after administration, P.C.: immediately after irradiation	Corneal epithelia	Paraquat, acridine orange, ethidium bromide, acrylamide, 4-nitroquinoline 1-oxide, 254 nm UV (1.0 J/cm^2)	UDS test	Tahara *et al.*, 2021a
Japanese white rabbits	Single ocular instillation	2 hr after administration	Corneal epithelial cells	Ethidium bromide, paraquat, methyl methane sulfonate, acrylamide, 4-nitroquinoline 1-oxide	Comet assay	Tahara *et al.*, 2021b
Japanese white rabbits	Single ocular instillation	0.5, 2, 4, 6, 24 hr after administration	Corneal epithelial cells	Ethidium bromide, methyl methane sulfonate, 4-nitroquinoline 1-oxide	Comet assay	Tahara *et al.*, 2022

UDS, unscheduled DNA synthesis test; P.C., positive control.

vitro studies were more than 10,000 times higher than the systemic exposure in clinical use (PMDA, 2007, 2010a, 2014a, 2014b). For the EYBELIS® ophthalmic solution (0.002%), the concentration that tested negative in the *in vitro* test was at least 20 times the clinically applicable concentration (eye drop concentration); therefore, genotoxicity to ocular tissues was judged to be of no concern and no additional toxicity testing was required. From the above, even if positive results were observed in the *in vitro* genotoxicity tests, the comprehensive genotoxic concern would be considered low in clinical use when no genotoxicity was observed in the *in vivo* tests, and there was a sufficient safety margin between the positive concentration in the *in vitro* test and the clinically applied concentration. However, if the concentrations that yielded positive results in the *in vitro* studies were close to the eye drop concentration, genotoxic concerns for ocular tissues must be explained. The UDS test using corneas is a method to evaluate these concerns.

Genotoxicity tests were conducted during the development of the oral or injectable drugs, and additional genotoxicity tests were conducted for a few ophthalmic drugs. Additional genotoxicity tests were performed during the development of two oral drugs because of the positive *in vitro* genotoxicity results. In both these products, *in vivo* UDS tests were performed on corneas after ocular instillation to assess the genotoxic potential of the ocular surface.

Carcinogenicity tests were conducted for only four of eight approved drugs with new active ingredients. Most drugs that have not been subjected to carcinogenicity tests were approved in 2010 or later. The lack of genotoxic concern in the *in vivo* studies, absence of neoplastic or preneoplastic lesions in chronic dose toxicity studies, and low systemic exposure following ocular instillation were the main reasons for forgoing carcinogenetic studies. Moreover, the ICH S1A guideline states that pharmaceuticals administered by the ocular route may not require carcinogenicity studies unless there is a cause for concern or significant systemic exposure (ICH, 1995). Thus, based on the weight of the evidence, it would be possible to explain the carcinogenic risk concern in humans without conducting carcinogenicity tests in rodents. Similarly, with EYBELIS® ophthalmic solution (0.002%), considering carcinogenic concerns in terms of TTC would be beneficial. Because TTC-based acceptable intake is considered safe for a lifetime of daily exposure, carcinogenic concerns would be significantly lower if the daily intake was below the TTC (PMDA, 2018). A tolerable intake of 1.5 μg/person/day based on TTC corresponds to an ophthalmic dose of 30 μL of 0.0025% ophthalmic solution in each eye once a day. Therefore, carcinogenicity tests must be conducted on a case-by-case basis to develop ophthalmic drugs.

No specific studies have been conducted on the development of intravitreal drugs. However, continuous surveys are required in the future because genotoxicity studies have been conducted for only one product.

The *in vivo* UDS tests using the cornea have been conducted for four drugs with new active ingredients and new administration routes. Although the UDS test using the cornea is not listed in the test guidelines, it is suggested that this test is the only test system that can appropriately assess the genotoxic potential of ocular surfaces. Moreover, the results of a survey of other test systems that assessed genotoxicity in ocular tissues, such as the comet assay with the cornea after ocular instillation and the comet assay with retina after intravitreal administration, have been reported. Only basal cells of the corneal epithelia undergo cell division (Jones and Marfurt, 1996), but not the corneal endothelia. In addition, the retina divides when it is damaged. Thus, ocular tissues contain actively dividing and non-dividing cells. The micronucleus test, the most widely conducted *in vivo* genotoxicity test, evaluates micronuclei, which are fragments of chromosomes that are not incorporated as nuclei into daughter cells during cell division. Micronuclei tests must be conducted to evaluate cells after division. However, the comet assay may be useful for the genotoxic evaluation of ocular tissues because it can be evaluated independently of the proliferative potential of the target organs. Various administration routes for ophthalmic drugs are known, including anterior chamber administration, sub-Tenon injection, and drug delivery system devices, in addition to ocular instillation and intravitreal administration. Therefore, it is important to establish evaluation systems suitable for target tissues for the administration route.

In this review, we surveyed genotoxicity and carcinogenicity testing packages for the development of ophthalmic drugs and discussed the differences in the application category. No major differences in genotoxicity test packages were observed by application category; however, the UDS test using the cornea following ophthalmic instillation was conducted for some products as a specific test for ophthalmic drugs. In the development of ophthalmic drugs, if a positive result is found in an *in vitro* genotoxicity test, the safety margin between the positive concentration and the clinically applicable concentration (eye drop concentration) is required for safety assessment. If the safety margin cannot be ensured, additional tests may support safety assessment.

Conflict of interest---- The authors of this manuscript are employees of Senju Pharmaceutical Co., Ltd.

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