Effect of antigen-dependent clearance on pharmacokinetics of anti-heparin-binding EGF-like growth factor (HB-EGF) monoclonal antibody

Noriyuki Kasai*, Yukitaka Yoshikawa, and Junichi Enokizono

R&D Division, Kyowa Hakko Kirin Co. Ltd., Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka, Japan

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Abbreviations: HB-EGF, heparin-binding EGF-like growth factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ADCC, antibody-dependent cellular cytotoxicity; SCID mouse, severe-combined immunodeficient mouse; IVIS, In Vivo Imaging System; SPR, surface plasmon resonance; LLOQ, lower limit of quantification

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family and is an important therapeutic target in some types of human cancers. KM3566 is a mouse anti-HB-EGF monoclonal antibody that neutralizes HB-EGF activity by inhibiting the binding of HB-EGF to its receptors. Based on the results of our pharmacokinetics study, a humanized derivative antibody, KHK2866, is rapidly cleared from serum and shows nonlinear pharmacokinetics in cynomolgus monkeys. In this study, we examined the antigen-dependent clearance of an anti-HB-EGF monoclonal antibody in vivo and in vitro in order to pharmacokinetically explain the rapid elimination of KHK2866. We revealed tumor size-dependent clearance of KM3566 in in vivo studies and obtained good fits between the observed and simulated concentrations of KM3566 based on the two-compartment with a saturable route of clearance model. Furthermore, in vivo imaging analyses demonstrated tumor-specific distribution of KM3566. We then confirmed rapid internalization and distribution to lysosome of KM3566 at a cellular level. Moreover, we revealed that the amounts of HB-EGF on cell surface membrane were maintained even while HB-EGF was internalized with KM3566. Recycled or newly synthesized HB-EGF, therefore, may contribute to a consecutive clearance of KM3566, which could explain a rapid clearance from serum. These data suggested that the rapid elimination in pharmacokinetics of KM3566 is due to antigen-dependent clearance. Given that its antigen is expressed in a wide range of normal tissue, it is estimated that the rapid elimination of KHK2866 from cynomolgus monkey serum is caused by antigen-dependent clearance.

Introduction

Epidermal growth factor (EGF) receptors and EGF family members represent promising targets for cancer therapy. Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family and is an important therapeutic target in some types of human cancers. HB-EGF binds to and activates both HER1 and HER4,1–3 and plays a pivotal role in many physiologic and pathologic processes via transduction of extracellular signals.4–6 HB-EGF has been reported to be involved in a number of pathological processes such as cardiac hypertrophy7 and tumorigenesis in ovarian cancer.8,9 It has also been shown that HB-EGF expression is significantly associated with the clinical outcome in ovarian cancer.10 Based on these evidence, HB-EGF is now considered to be a therapeutic target in human disease.

KM3566 is a mouse anti-HB-EGF monoclonal antibody (IgG1/κ) that neutralizes HB-EGF activity by inhibiting the binding of HB-EGF to its receptors.11 The mouse–human chimeric counterpart for KM3566 (cKM3566) induces dose-dependent antibody-dependent cellular cytotoxicity (ADCC) against cancer cells that express HB-EGF in vitro, and significantly inhibited tumor growth in severe combined immunodeficient mice inoculated with MCAS or ES-2 human ovarian cancer cells.11 The humanized derivative, KHK2866, was generated as a drug candidate for cancer therapeutics.11

In the course of the development of KHK2866, we investigated the pharmacokinetics of KHK2866 after a single intravenous administration to cynomolgus monkeys. As a result, the mean half-life values at 1 mg/kg were 1.50 d (n = 3, male) and 1.51 d (n = 3, female), and those at 100 mg/kg were 3.98 (n = 3, male) and 4.08 d (n = 3, female), respectively. The mean total clearance values at 1 mg/kg were 13.9 mL/day/kg (n = 3, male) and 15.9 mL/day/kg (n = 3, female), and those at 100 mg/kg were 9.03 mL/day/kg (n = 3, male) and 9.76 mL/day/kg (n = 3, female), respectively. KHK2866 administered intravenously to cynomolgus monkey exhibited rapid elimination from serum and nonlinear pharmacokinetics at doses of 1 and 100 mg/kg. No anti-KHK2866 antibody was detected in the
pharmacokinetic study. A previous study indicates that HB-EGF is expressed in normal human tissues like lung, liver, kidney, pancreas, and ovary. Moreover, HB-EGF distribution pattern of normal human tissues is similar to that of normal cynomolagus monkey tissues based on our internal study (data not shown). Therefore, it is possible that rapid elimination of KHK2866 from cynomolagus monkey serum is caused by antigen-dependent clearance.

Many therapeutic antibodies were reported to show nonlinear pharmacokinetics and increased clearance in low dosage. It is known that antigen-mediated clearance is largely responsible for the nonlinear pharmacokinetics and increased clearance in some therapeutic antibodies. Furthermore, elimination of anti-EGFR monoclonal antibodies by binding to normal tissues where EGFR is highly expressed has been hypothesized as a significant clearance route.

In this study, we examined antigen-dependent clearance of an anti-HB-EGF monoclonal antibody in vivo and in vitro in order to explain the pharmacokinetics of the rapid elimination of KHK2866.

Results

Pharmacokinetics of KM3566 after a single intravenous administration in control SCID mice and xenograft mice bearing variously sized tumors

The serum concentration-time curves of KM3566 intravenously administrated to control SCID mice and xenograft mice bearing variously sized tumors are shown in Figure 1A. The serum concentration of KM3566 was below the LLOQ (400 ng/mL) at the time point of 13 d after dosing in 200, 400, and 600 mm\(^3\) groups. The mean total clearance (CL\(_{\text{total}}\)) of each group were 8.24, 14.0, 16.9, 19.9, and 29.1 mL/day/kg in 0 (control, n = 3), 100 (n = 3), 200 (n = 3), 400 (n = 3), and 600 (n = 1) mm\(^3\) groups, respectively. The relationship between CL\(_{\text{total}}\) and tumor volume is shown in Figure 1B. The CL\(_{\text{total}}\) values had a good linear correlation with tumor volume, suggesting tumor-dependent clearance of KM3566.

Pharmacokinetics of KM3566 after a single intravenous administration of three different doses in control SCID mice and xenograft mice

In Figure 2A, KM3566 serum concentrations observed after single i.v. dosing of 0.1, 1, or 10 mg/kg in control and xenograft mice are plotted vs. time. The serum concentrations of KM3566 were below the LLOQ (30 ng/mL) at 624 h in xenograft 1 mg/kg group and 336 h in xenograft 0.1 mg/kg group. The serum concentrations of KM3566 for the xenograft 0.1 mg/kg group were not available due to the euthanasia of mice at 504 h and thereafter. Pharmacokinetic parameters (CL\(_{\text{total}}\), V\(_{\text{ss}}\), and t\(_{1/2}\)) obtained by non-compartmental analysis are summarized in Table 1. Based on the results, there was a clear difference in PK between control and xenograft mice, suggesting that xenograft mice should have another clearance route (i.e., antigen-dependent clearance). In fact, comparison between three groups given dosages of 0.1, 1, 10 mg/kg in xenograft mice revealed that CL\(_{\text{total}}\) decreased and t\(_{1/2}\) was prolonged with increasing dose, whereas control mice showed approximately linear pharmacokinetics.

For additional analysis, we plotted the simulated time course and determined other PK parameters based on the two-compartmental model for control mice and the two-compartment with a saturable route of clearance model for xenograft mice as described in the Materials and methods (Fig. 2 and Table 2). A good fit for each dosing of both control and xenograft mice was obtained between the observed and simulated concentrations.

Imaging analysis for distribution of KM3566 after a single intravenous administration in xenograft mice

In Vivo Imaging System (IVIS) images of KM3566 distribution in live, anesthetized xenograft mice were taken on the same conditions (e.g., exposure, power) at 15 min, 5, and 24 h after a single intravenous administration. As shown in Figure 3, tumor-specific distribution of KM3566 was observed at 5 h after administration. Clearer distribution of KM3566 was observed at 24 h after administration.

Internalization and distribution to lysosome of KM3566 analyzed by confocal microscopy

Internalization and distribution to lysosome of KM3566 were analyzed by IN Cell Analyzer 6000 (Fig. 4). We performed double staining experiments using Lysotracker green DND-26, lysosome-specific marker in order to examine the co-localization of KM3566 with lysosome. Typical images are presented in Figure 4. Internalization was clearly observed at 5 h and thereafter based on the images obtained in the analysis. Furthermore, co-localization of KM3566 with lysosome was clearly observed at 24 h in broad areas of the image.

Fluctuation of antigen amounts on the cell surface membrane after addition of KM3566 to MCAS cells

Fluctuation of antigen amounts on the cell surface membrane after addition of KM3566 to MCAS cells was analyzed by a flow cytometer (Fig. 5). Relative values to the initial mean fluorescent intensities decreased sharply to 0.7 at 0.5 h after incubation with KM3566, and then became stable between approximately 0.7 and 0.8 until the end of the experiment (48 h).

Discussion

Epidermal growth factor (EGF) receptors and EGF family members represent promising targets for cancer therapy. Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family and is an important therapeutic target in some types of human cancers. KM3566 is a mouse anti-HB-EGF monoclonal antibody (IgG1/k) that neutralizes HB-EGF activity by inhibiting the binding of HB-EGF to its receptors. The mouse–human chimeric counterpart for KM3566 (cKM3566) induces dose-dependent ADCC activities against cancer cells expressing HB-EGF in vitro, and significantly inhibited tumor growth in severe combined immunodeficient mice inoculated...
with MCAS or ES-2 human ovarian cancer cells. The humanized derivative, KHK2866, was generated as a drug candidate for cancer therapeutics.

Based on the study results of the pharmacokinetics of KHK2866 after a single intravenous administration to cynomolgus monkeys, KHK2866 is rapidly cleared from the serum and shows nonlinear pharmacokinetics as described in Introduction. It is confirmed that the rapid clearance was not due to the production of anti-human IgG antibodies. Taking the expression patterns of HB-EGF (i.e., lung, liver, kidney, pancreas, and ovary) into account, it is possible that rapid elimination of KHK2866 from cynomolgus monkey serum is caused by

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**Figure 1.** (A) Pharmacokinetics of KM3566 after a single intravenous administration in control SCID mice and xenograft mice bearing variously sized tumors. (B) The relationship between $\text{CL}_{\text{total}}$ and tumor volume. (A) A single intravenous injection of KM3566 at a dose of 1 mg/kg was administered to male control SCID mice and xenograft mice bearing variously sized tumors. In the control and 100 mm$^3$ groups, blood samples were collected at 1, 2, 5, 7, and 12 d after administration, and blood samples were collected at 1, 2, 5, 7, and 13 d after administration in other groups. The concentrations of KM3566 in serum were determined by Biacore SPR assay method. Each point of 0, 100, 200, and 400 mm$^3$ tumor-bearing mice represents the mean ± SD ($n=3$), and each point of 600 mm$^3$ represents the concentration from one mouse. The serum concentration of KM3566 was below the LLOQ (400 ng/mL) at the time point of 13 d after dosing in 200, 400, and 600 mm$^3$ groups. (B) The $\text{CL}_{\text{total}}$ values had a significant correlation with tumor volume.
antigen-dependent clearance. In this study, we examined antigen-dependent clearance of an anti-HB-EGF monoclonal antibody in vivo and in vitro in order to explain the pharmacokinetics of the rapid elimination of KHK2866.

First, we investigated the pharmacokinetics of KM3566 after a single intravenous administration in control SCID mice and xenograft mice bearing variously sized tumors. The serum concentration-time curves of KM3566 (Fig. 1A) shows that KM3566 was cleared from serum more rapidly in xenograft mice as compared with control SCID mice, indicating the presence of an additional route of clearance in xenograft mice. Furthermore, the CL_total values had a positive linear correlation with tumor volume (Fig. 1B). These results strongly suggested that tumor (i.e., antigen)-dependent clearance had a substantial effect on KM3566 pharmacokinetics. Second, we investigated in-depth pharmacokinetics in order to examine how the antigen affects the clearance of KM3566. As shown in Figure 2A, the plots of KM3566 serum concentrations observed after single i.v. dosing of 0.1, 1, or 10 mg/kg in control and xenograft mice vs. time revealed that there was a clear difference in pharmacokinetics between control and xenograft mice at a glance. Moreover, pharmacokinetic parameters (CL_total, Vss, and t1/2) obtained by non-compartmental analysis demonstrated nonlinear and approximately linear pharmacokinetics in xenograft and control mice, respectively (Table 1). For additional analysis, we plotted the simulated time course and determined other PK parameters based on the two-compartmental model for control mice and the two-compartment with a saturable route of clearance model for xenograft mice as described in the Materials and methods (Fig. 2 and Table 2). A good fit for each dosing of

Table 1. PK parameters of KM3566 after a single intravenous administration in control SCID mice and xenograft mice

| Dose (mg/kg) | CL_total (mL/h/kg) | Vss (mL/kg) | t1/2 (h) |
|-------------|-------------------|-------------|----------|
| Xenograft   |                   |             |          |
| 0.1         | 1.66 ± 0.24       | 121 ± 9     | 56.3 ± 12.4 |
| 1           | 0.885 ± 0.188     | 103 ± 11    | 81.1 ± 9.2 |
| 10          | 0.397 ± 0.063     | 93.5 ± 3.3  | 175 ± 35  |
| Control     |                   |             |          |
| 0.1         | 0.405 ± 0.018     | 172 ± 13    | 304 ± 18  |
| 1           | 0.289 ± 0.009     | 126 ± 3     | 312 ± 17  |
| 10          | 0.240 ± 0.010     | 93.9 ± 5.5  | 282 ± 21  |

PK parameters (CL_total, Vss, and t1/2) were obtained by non-compartmental analysis. Results are the mean ± SD of three or four animals per group.
both control and xenograft mice was obtained between the observed and simulated concentrations, which strongly supported the hypothesis that antigen-dependent clearance is an additional saturable route of elimination. Considering the $K_m$ value was 18.0 $\mu$g/mL, the presence of an additional route of clearance should become dominant at much lower concentrations (i.e., all the time points at 0.1 mg/kg dosage), whereas it is negligible at much higher concentrations (i.e., some initial time points at 10 mg/kg dosage). We measured the affinity ($K_D$ value) between recombinant human HB-EGF and KM3566 by Biacore SPR analysis. As a result, the $K_m$ value ($=120$ nmol/L) was totally different from the $K_D$ value (1.61 nmol/L) of KM3566 (data not shown). We speculate that the gap between the two values is due to the difference in KM3566 concentrations between serum and tumor tissues. The difference between the $K_m$ and $K_D$ values can be explained by the inability of serum KM3566 to fully reach tumor tissues. It is known that tissue vascularization is immature in tumor tissues and other factors such as physical hindrance of the collagen network in the tumor matrix and enhanced interstitial pressure can result in delayed biodistribution of drugs. The difference in the $K_m$ and $K_D$ values can also be due to their different definition; the $K_D$ value is calculated from $k_{on}$ and $k_{off}$ values only, whereas the elimination rate after binding to the antigen is also considered for calculation of the $K_m$ value. The elimination is not negligible in the case of KM3566 pharmacokinetics, therefore, the difference in the $K_m$ and $K_D$ values is expected. Furthermore, we conducted in vivo imaging analysis for distribution of KM3566 after a single intravenous administration in xenograft mice. As shown in Figure 3, KM3566 accumulated in the tumor area specifically even after 5 h post administration and more defined distribution was observed at 24 h post. These results suggest that KM3566 predominantly distribute to tumor tissues and is subjected to antigen-dependent clearance.

We then conducted in vitro studies in order to confirm whether the results obtained from the in vivo studies could be extrapolated from cellular- and molecular- analysis. First, internalization and distribution to lysosome of KM3566 were analyzed by confocal microscopy. Figure 4 showed that internalization of KM3566 was clearly observed at 5 h and thereafter. Furthermore, co-localization of KM3566 with lysosome was clearly observed at 24 h in broad areas of the images. Based on the results, antigen-dependent clearance predicted in KM3566 pharmacokinetics in xenograft mice was considered to be caused by internalization and distribution to lysosome, followed by catabolism. Second, fluctuation of antigen amounts on the cell surface membrane after addition of KM3566 to MCAS cells was analyzed by a flow cytometer. As a result, relative values to the initial mean fluorescent intensities decreased sharply to 0.7 at 0.5 h after incubation with KM3566, and then became stable between approximately 0.7 and 0.8 until the end of the experiment (Fig. 5). These results suggested that the amounts of HB-EGF on cell surface membrane were maintained even while HB-EGF was internalized with KM3566. Recycled or newly synthesized HB-EGF, therefore, may contribute to a consecutive clearance of KM3566, which could explain a rapid clearance from serum. In general, the dynamics of a therapeutic antibody and its antigen molecule may affect antibody pharmacokinetics and anti-tumor efficacy. In the case of our anti-HB-EGF antibody, it is estimated that large amounts of antibodies
would be needed for blockade of signaling due to rapid antibody clearance and constant antigen expression on the cell surface. The traits of the antibody and antigen could be appropriate for antibody drug conjugate (ADC) format because ADC needs to be internalized as much as possible for sufficient anti-tumor efficacy.

In summary, the in vitro and in vivo results obtained in this study suggested that the rapid elimination in pharmacokinetics of an anti-HB-EGF monoclonal antibody, KM3566, is due to antigen-dependent clearance. Given that its antigen is expressed in a wide range of normal tissue, it is estimated that rapid elimination of KHK2866 from cynomolgus monkey serum is caused by antigen-dependent clearance.

Materials and methods

Materials
Recombinant human HB-EGF was purchased from R&D Systems (catalog number 259-HE-050). KM3566, a mouse anti-HB-EGF monoclonal antibody was obtained by immunization of recombinant human HB-EGF to HB-EGF null mice as described in the previous report. KHK2866, a humanized derivative which has the same epitope of KM3566, showed high affinity for human and monkey HB-EGF, but no cross-reactivity to rat and mouse HB-EGF (data not shown). It is thus suggested that KM3566 has the same cross-reactivity to HB-EGF of each species as KHK2866. KM3579, a mouse monoclonal anti-HB-EGF antibody with a different epitope and its Alexa647-labeled antibody were also obtained from Kyowa Hakko Kirin. KM4041, a mouse monoclonal anti-KHK2866 idiotypic antibody, and its ruthenylated antibody were obtained from Kyowa Hakko Kirin.

Other chemicals and reagents were of the highest grade and purchased from local commercial sources.

Mice and cell lines
C.B-17/scid Jcl [severe-combined immunodeficient (SCID)] mice were purchased from CLEA Japan, Inc. MCAS (JCRB0240), a human ovarian cancer cell line, was purchased from Japanese Collection of Research Bioreresources.

Animal experiments
All of the in vivo experiments were performed in conformity with institutional guidelines in compliance with national laws and policies.
Pharmacokinetics of KM3566 after a single intravenous administration in control SCID mice and xenograft mice bearing variously sized tumors

A single intravenous injection of KM3566 at a dose of 1 mg/kg was administered to male control SCID mice and xenograft mice bearing variously sized tumors. Tumor-bearing mice were prepared by s.c. injection of MCAS cells (2.5 × 10⁶) into 7-wk-old male SCID mice. A single intravenous injection of KM3566 to xenograft mice was conducted after tumors reached approximately 100, 200, 400, and 600 mm³. One mouse bearing 600 mm³ tumor and three mice bearing 0 (control), 100, 200, or 400 mm³ tumor each were examined for this pharmacokinetic study. The tumor volume was calculated according to the following formula:

Tumor volume (mm³) = 0.5 × (major diameter) × (minor diameter)²

In the control and 100 mm³ groups, blood samples were collected from the tail vein at 1, 2, 5, 7, and 12 d after administration. The serum samples obtained from collected blood were stored frozen until analysis. In the other groups, blood samples were collected from the tail vein at 1, 2, 5, 7, and 13 d after administration and serum was stored in the same way above.

The concentrations of KM3566 in serum were determined by Biacore SPR assay method described below. Pharmacokinetic parameters were obtained by non-compartmental analysis of a series of serum concentration data for individual animal using Phoenix™ WinNonlin® (version 6.1; Pharsight Corporation).

Pharmacokinetics of KM3566 after a single intravenous administration of three different doses in control SCID mice and xenograft mice

A single intravenous injection of KM3566 at a dose of 0.1, 1, and 10 mg/kg was administered to male control SCID mice and xenograft mice. Xenograft mice were prepared by s.c. injection of MCAS cells (2.5 × 10⁶) into 7-wk-old male SCID mice. When mean tumor sizes reached about 100 mm³, mice were randomized into 3 groups (0.1 and 10 mg/kg: 3 mice/group, 1 mg/kg: 4 mice/group) based on the tumor sizes. The tumor volume was calculated according to the formula described above. Treatments were initiated from the day of grouping and the day of the grouping was set as day 0. In the same way, control SCID mice were randomized into 3 groups (3 mice/group) based on the body weights and pharmacokinetic of KM3566 in control SCID mice was also examined. Blood samples were collected from the tail vein at 1, 5, 24, 48, 125, 168, 336, 504 and 624 h after administration. The serum samples were stored frozen until analysis.

The concentrations of KM3566 in serum were determined by electrochemiluminescent immunoassay method described below. Pharmacokinetic parameters (CLtotal, Vss, and t1/2) were obtained by non-compartmental analysis of a series of serum concentration data for individual animal using Phoenix™ WinNonlin® (CLtotal: total clearance, Vss: steady-state distribution volume, t1/2: serum half-life). Other pharmacokinetic parameters were obtained as follows. First, a two-compartmental model curve-fit was used to determine pharmacokinetic parameters (k12, k21, and kconst) of KM3566 in control SCID mice after a single intravenous administration of three different doses (k12: rate constant for transfer from central compartment to peripheral compartment, k21: rate constant for transfer from peripheral compartment to central compartment, and kconst: rate constant for first-order elimination from central compartment). In the analysis, simulated KM3566 serum concentrations were calculated in an Excel 2003 (Microsoft) worksheet by using the following equations where V₁ represents volume of distribution in the central compartment. Model parameters were optimized by minimizing the 1/Y²-weighted sum-of-squares using the Excel’s Solver add-in.

Next, in the case of analysis for tumor-bearing xenograft mice, the second route of clearance, which is saturable, was introduced to describe the nonlinear clearance behavior (Fig. 2B). The two-compartmental model with saturable route of clearance curve-fit was used to determine maximum rate of elimination (Vmax) and antibody concentration for half-maximum elimination (Km) of KM3566 in xenograft mice after a single intravenous administration of three different doses. In the analysis, simulated KM3566 serum concentrations were calculated by using the following equation. Model parameters

Table 2. Model-based PK parameters of KM3566 after a single intravenous administration in control SCID mice and xenograft mice

| k₁₂ | k₂₁ | kconst | Vmax | Km |
|-----|-----|--------|------|----|
| 0.0544 (h⁻¹) | 0.0445 (h⁻¹) | 0.00519 (h⁻¹) | 301 (ng/mL/h) | 18.0 (μg/mL) (≈120 nmol/L) |

Additional PK parameters were determined based on the two-compartmental model for control mice and the two-compartment with a saturable route of clearance model for xenograft mice.
were optimized as described above.

\[
C(t + \Delta t) = C(t) \cdot e^{-\beta(t) \cdot \Delta t}
\]

\[
\beta(t) = \frac{k_{k2} + k_{c2} + k_{c}}{-2(\sqrt{(k_{k2} + k_{c2} + k_{c})^2 - 4k_{k2} \cdot k_{c2}} + k_{c})}
\]

\[
k_{c} = k_{\text{const}} + \frac{V_{\text{max}}}{K_{m} + C(t)}
\]

**Imaging analysis for distribution of KM3566 after a single intravenous administration in xenograft mice**

Imaging analysis for distribution of KM3566 after a single intravenous administration in xenograft mice was performed with IVIS (PerkinElmer). Xenograft mice were prepared by s.c. injection of MCAS cells (2.5 \times 10^6) into 5-wk-old male SCID mice. A single intravenous injection of XenoLight CF750 (Caliper, catalog number 125674) labeled KM3566 at a dose of 1 mg/kg was conducted after tumor sizes reached approximately 200–400 mm^3. MCAS tumor-bearing mice were prepared as described above. IVIS images of KM3566 distribution in live, anesthetized xenograft mice were taken at 15 min, 5, and 24 h after administration.

**Determination of KM3566 concentration in serum samples by SPR method**

Serum KM3566 concentrations in the pharmacokinetic study of a single intravenous administration in control SCID mice and xenograft mice bearing variously sized tumors were determined using a Biacore® T100 instrument (GE Healthcare Japan). To detect concentration-dependent binding of KM3566 to HB-EGF, recombinant human HB-EGF was immobilized on a CM5 sensor chip by the amine coupling method to obtain approximately 1100 RU. 10-Fold diluted samples including KM3566 were injected over the HB-EGF-immobilized chip at 30 μL/min for 60 s. Response units 160 s after the end of injection were used for concentration analysis. After each experiment, regeneration was performed using 10 mM Glycine-HCl (pH 1.5). The quantification range was 400–50000 ng/mL.

**Internalization and distribution to lysosome of KM3566 analyzed by confocal microscopy**

Internalization and distribution to lysosome of KM3566 were observed in the immunofluorescence analysis. MCAS cells were seeded in a 96-well culture plate and cultured overnight to attach. The cells were incubated with Alexa647-labeled KM3566 diluted with Minimum Essential Medium (MEM; Life technologies, catalog number 11095–080) including 1 v/v% Antibiotic-Antimycotic (Life technologies, catalog number 15240–062) and 20 v/v% FBS for 0 (control), 0.5, 5, 24 h at 37°C. Lysotracker green DND-26 (Life technologies, catalog number L-7526) was added to cells 1 min before the end of incubation. Cells were washed once after the incubation, and then analyzed by confocal microscope mode of IN Cell Analyzer 6000 (GE Healthcare Japan).

**Fluctuation of antigen amounts on the cell surface membrane after addition of KM3566 to MCAS cells**

Fluctuation of antigen amounts on the cell surface membrane after addition of KM3566 to MCAS cells was analyzed by a flow cytometer. MCAS cells were seeded in a 24-well culture plate and cultured overnight to attach. The cells were incubated with excessive amounts of KM3566 diluted with MEM including 1 v/v% Antibiotic-Antimycotic and 20 v/v% FBS for 0 (control), 0.5, 1, 2, 4, 6, 24, and 48 h at 37°C. The cells were then rinsed twice and incubated with Alexa647-labeled KM3579 which has a different epitope of human HB-EGF on ice for 1 h. The cells with antigen-bound Alexa647-labeled KM3579 were analyzed by FACSCanto™ II (BD Biosciences) after the cells were washed once.

**Disclosure of potential conflicts of interest**

All authors are current employees of Kyowa Hakko Kirin, Co. Ltd.

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