Tropical Journal of Pharmaceutical Research September 2018; 17 (9): 1885-1891
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i9.30

Original Research Article

Evaluation of pharmacokinetics and toxicology of biosimilar APZ001 antibody in *Macaca cynomolgus*

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Sent for review: 31 May 2018 *Revised accepted: 20 August 2018*

Abstract

Purpose: *To compare the pharmacokinetics of APZ001 antibody with those of cetuximab (Erbitux®) and to evaluate the toxicology of the former.*
Methods: *To evaluate cetuximab's biosimilar APZ001, Crl:CD1(ICR) (CD-1) mice and Macaca fascicularis (cynomolgus monkey) were chosen for the studies on acute toxicity, chronic toxicity, pharmacokinetics in chronic toxicity and immunogenicity toxicity. The study also compared the pharmacokinetic parameters of APZ001 with those of cetuximab upon single and multiple drug administrations in cynomolgus monkeys.*
Results: *Pharmacokinetic parameters including maximum concentration (C_{max}) and time to attain maximum drug concentration (T_{max}), clearance rate and apparent volume of distribution of APZ001 were compared with those of cetuximab in both single and multiple administration studies. Difference of pharmacokinetics from weekly administration of APZ001 and cetuximab in cynomolgus monkeys was insignificant ($p > 0.05$), with relative bioavailability of 116.9 %. Both APZ001-treated and cetuximab-treated CD-1 mice showed the same level of food intake and body weight. Hematological and serological data were similar from APZ001 antibody and cetuximab treatments, so were the acute and chronic toxicity. Weekly transfusion of APZ001 did not alter its pharmacokinetic parameters. The administered drug was hardly detected in the serum in the 31st and 37th week of recovery; no accumulation of drug was observed upon withdrawal.*
Conclusion: *APZ001 has extremely similar characteristics as cetuximab in terms of pharmacokinetics and toxicity.*

Keywords: *Cetuximab, Pharmacokinetics, Acute toxicity, Chronic toxicity, Immunogenicity, Biosimilar*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Aberrant overexpression and malfunction of epidermal growth factor receptor (EGFR) have been observed in many cancers, of which proliferation, apoptosis, angiogenesis and metastasis were mediated by it [1,2]. Upon EGF binding, EGFR forms hetero- or homo-dimers, leading to auto-phosphorylation and subsequently activating intracellular signaling

transduction [3,4]. Suppressing cancer EGFR signaling via anti-EGFR monoclonal antibody (mAb) blocking is, therefore, an attractive therapeutic strategy [4].

Several mAbs-targeting cancer therapies have been approved by the Food and Drug Administration (FDA), including Erbitux (cetuximab) and Vectibix (panitumumab), which are used to treat colorectal cancer (CRC) and/or squamous cell carcinoma of the head and neck (SCCHN) by targeting EGFR [5,6]; Herceptin (trastuzumab) and pertuzumab, on the other hand, are used to treat gastric cancers by targeting EGFR2 [7,8]. It has been reported that a combined treatment of cetuximab and radiotherapy of the SCCHN increased the survival rate from 36 to 45 % during the phase III clinical trial (p = 0.018).

Due to the economic consideration, however, use of Erbitux and Herceptin are limited in the less developed countries. This work focused on developing a substitute of Erbitux and Herceptin for a more affordable therapy option. The cetuximab biosimilar antibody APZ001, of which the protein sequence and biological functions bear similarity to cetuximab, was investigated, with its preclinical pharmacokinetics and pharmacovigilance evaluated.

EXPERIMENTAL

Reagents and drugs

Cetuximab (used as the positive control) was purchased from Merck Serono (lot: 219265, imported drug license: S201300041). Saline buffer (0.9 %) was purchased from Kelun Pharmaceutical Co. Inc. (lot: B120518 F1); pentobarbital sodium from Sinopharma Chemical Reagent Co. (#090205); ketamine hydrochloride from Shenyang Veterinary Medicines Co. (#20120501); serum quality control from Sysmex Inc. (#30010802); leucocyte hemolysin and basophil hemolysin from Sysmex Inc. (#R2021, #R2013). ELISA kits for IgA, IgE, IgG, and IgM were purchased from Fanbang Biotech Company (#20130402B) and were used according to the manufacturer's protocol. HRP-labeled secondary antibody was purchased from Jackson (#109-035-088) and TMB buffer was purchased from Neogen (#308176).

Quantification of serum antibody concentration

The serum concentration of cetuximab and APZ001 antibody was quantified by ELISA assay. 96-well plate coated with EGFR (50 µL, 0.25 µg/mL) was incubated at 4 ℃ overnight and was then washed with PBS.

The plate was blocked with BSA (2 %) at 25 ℃ for 2 h, after which the serum (100 µL) was added and incubated at 25 ℃ for 2 h. Subsequent to the PBS wash was HRP-labeled anti-human secondary antibody added and together incubated at 25 ℃ for 1 h. Results were visualized by TMB buffer (100 µL) and were then terminated by the Stop solution (50 µL). The absorbance was read by spectrophotometer (BioTek synergy H1) and was then processed and analyzed.

Animal ethics and welfare

The use of cynomolgus monkeys (*Macaca fascicularis*) was licensed by the Institutional Animal Care and Use Committee of Guangdong Lewwin Pharmaceutical Research Institute (approval no. SYXK, Guangdong, 2009-0099). Cynomolgus monkeys and forage were purchased from Guangdong Landau Biotechnology Ltd. In each 140 × 90 × 90-cm stainless steel cage kept two monkeys. They were kept at a temperature range from 16.3 to 19.7 °C with the relative humidity around 58.3 ~ 69.2 %. Fresh air was ventilated 8 - 10 times per hour; the artificial light-dark cycle was 12:12. The monkeys were injected with ketamine 8 mg/kg hydrochloride (0.1 mL/kg) for anesthesia. Pentobarbital sodium (30 mg/kg, 1.0 mL/kg,i.v.) was then injected to execute euthanasia. All animal-related experiments strictly followed the General principles for non-clinical safety technical reviews of therapeutic biologics and the Guide for the Care and Use of Laboratory Animals [9,10].

Pharmacokinetic studies

Thirty cynomolgus monkeys were randomly divided into five groups, with three males and three females in each group. Groups 1 to 3 received 7.5, 25 and 75 mg/mL of cetuximab biosimilar APZ001, respectively; group 4 received 75 mg/kg cetuximab for single pharmacokinetics analysis. APZ001 was administered for 4 weeks at a concentration of 25 mg/kg for multiple dose pharmacokinetics analysis. Blood samples were collected from groups 1 to 4 at 10 min, 20 min and 30 min (during administration); 1 h (administration endpoint); and at 4 h, 8 h, 24 h, 3 d, 5 d, 7 d, 9 d, 11 d, 14 d, 17 d, 21 d, 24 d and 28 d (after drug administration).

For group 5, cynomolgus monkeys received APZ001 weekly with their blood samples

collected before the drug treatment (0 min), then at 10 min, 20 min and 30 min (during administration); 1 h (administration endpoint); and at 4 h, 8 h, 24 h, 3 d and 5 d (after drug administration). After the fourth weekly drug administration, blood samples were collected at 9 d, 11 d, 14 d, 17 d, 21 d, 24 d, 28 d, 30 d, 32 d, 35 d, 38 d, 42 d, 45 d and 49 d.

Toxicity test

To test the toxicity of biosimilar APZ001, 50 cynomolgus monkeys were randomly divided into 5 groups with sexual equality: group A, negative control; group B, cetuximab positive control; group C, low concentration of APZ001 administered; group D, medium concentration of APZ001 administered; group E, high concentration of APZ001 administered. Drugs were injected intravenously weekly after the initial administration, of which the concentration was at 12 mL/kg (week 1); the drugs were then delivered at 7.5 mL/kg in the following experiment weeks (weeks 2-26).

Leukocyte differential count, bone marrow white blood cell classification, and immunoglobin protein analysis were also performed. The clinical condition of the animal was also recorded daily, including the symptoms, start time, severity, duration and reversibility of toxicity. Weeks 5 and 11 were set as the recovery periods.

Immunogenicity test

Blood samples were collected for immunogenicity examination during the chronic toxicity test. IgA, IgE, IgG and IgM were measured by ELISA a week before the administration and then at week 4, 13, 26, 31 and 37. Anti-drug antibody was determined a week before the administration and then at week 1, 2, 4, 6, 8, 10, 12, 20, 26, 28, 31 and 37. Leukocyte differential count, bone marrow white blood cell classification and immunoglobin protein analysis were performed to monitor the change in the immunogenic indicators.

Statistics analysis

All statistics analysis was processed with Excel software. Pairs of samples were compared by F-test for equality of variances before using the t-test or U-test. Watson LIMS v.7.3.0.01 (Thermo Scientific Inc.) was used for serum drug concentration analysis. WinNonLin v 5.2.1 (Pharsight Inc.) software was used to calculate pharmacokinetic parameters.

RESULTS

Pharmacokinetics of APZ001 and cetuximab administered as a single dose

C_{max} and AUC values increased along with the administration concentration: the C_{max} of 7.5, 25 and 75 mg/kg APZ001 were 183.08 ± 10.89, 642.98± 49.97 and 2132.31 ± 229.53 μg/mL, respectively, showing no statistically significant difference ($p > 0.05$); the AUC_{inf} were 9661.09 ± 1250.66, 53608.67 ± 7852.95, and 183350.05± 20360.84 h•μg/mL, respectively. Significant differences among three groups were noted ($p < 0.05$, Table 1) upon 7.5 and 25 mg/kg administration; the drug concentration showed a non-linear decrease, whereas the decrease was linear for the 75 mg/kg administration. The terminal elimination half-life of the low, medium, and high dosage groups were 6.29 ± 4.6, 69.11 ± 21.43, and 50.92 ± 46.69 h, respectively. The clearance rate (CL/F) was not significantly different between cetuximab and APZ001 at 25 mg/kg (Figure 1, $p > 0.05$) and was the same for the medium and high dose groups ($p > 0.05$, Table 1). The apparent volume of distribution (Vd) was also similar among the groups ($p > 0.05$, Table 1).

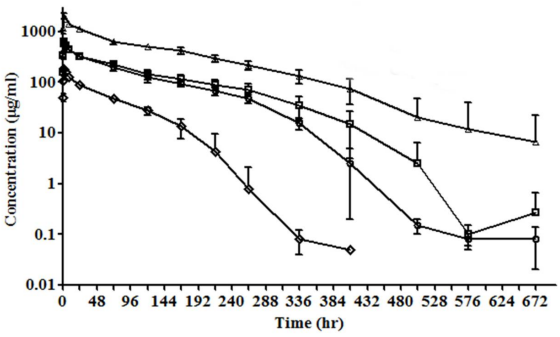

Figure 1: Pharmacokinetics of APZ001 and cetuximab after single dose administration. ◊represents 7.5 mg/kg APZ001; □ represents 25 mg/kg APZ001; Δ represents 75 mg/kg APZ001; ○ represents 25 mg/kg cetuximab. Comparison of equivalent doses between APZ001 and cetuximab did not show significant differences in terms of pharmacokinetic parameters ($p > 0.05$). Data were reported as mean ± SD

Pharmacokinetics of APZ001 and cetuximab administered as multiple doses

In group 5, administration of APZ001 at 25 mg/kg was repeated weekly. The T_{max}s of initial and terminal drug transfusion were 0.67 ± 0.26 and 0.92 ± 0.20, respectively. Initial and terminal C_{max}s were 668.61 ± 56.05 and 730.56 ± 114.22 μg/mL; AUCs(0-t) were 37543.2 ± 4484.23 and 59742.48 ± 33016.40 h•ng/mL; and AUC_{inf}s were

51676.29 ± 10262.62 and 59799.5 ± 33122.55 h•μg/mL, respectively. Initial and terminal elimination T1/2s were 84.81 ± 20.67 and 57.99 ± 11.65 h; clearance rates were 0.50 ± 0.10 and 0.60 ± 0.48 mL/h/kg; and Vd were 59.19 ± 8.84 and 47.01 ± 28.19 mL/kg, respectively.

Figure 2 shows the serum drug concentrations at each collection and the mean value of the group (n = 6). After four administrations, the drug accumulation ratio was 1.18 ± 0.07. Pharmacokinetic parameters of the initial and terminal administrations were not significantly statistically different. The serum drug concentration quickly reached a stable level upon repeated administration with no drug accumulation detected. In conclusion, the multiple administrations resulted in similar outcomes for both APZ001 and cetuximab (p > 0.05); the pharmacokinetic parameters also showed little difference (p > 0.05) during the following treatment, with relative bioavailability 116.9 %.

Toxicity

During the 26-week chronic drug administration, serum concentrations of APZ001 or cetuximab were tested (Figure 3) and biomarkers for liver function and kidney function were analyzed afterwards. Chronic treatment with 38/24 mg/kg cetuximab, 38/24 mg/kg APZ001 or 120/75 mg/kg APZ001 increased the levels of albumin (ALB) and globulin (GCB) but decreased the A/G ratio, which recovered in the first recovery period. Several doses increased the levels of alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), glutamic dehydrogenase (GLDH) and aspartate amino transferase (AST). GLDH level returned to the baseline after two recovery periods, while other biomarkers only after one. Although the levels of liver biomarkers were found to increase, no significant changes were observed in liver weight or liver pathological examination. The 38/24 mg/kg dose cetuximab.

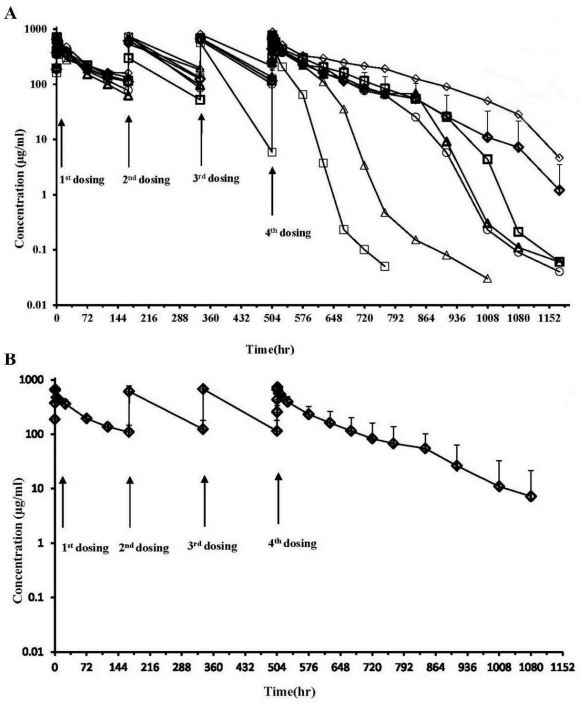

Figure 2: Pharmacokinetics of APZ001 and cetuximab after multiple administrations. For the 48-day multi-transfusion experiment, 25 mg/kg APZ001 was administered weekly. The APZ001 serum concentration of each monkey (A). □represents the 147th monkey;Δ represents the 149th monkey; ○ represents the 27th monkey; ◊represents the 41st monkey; □represents the 43rd monkey; **Δ**represents the 45th monkey; ◊represents the mean concentration. Mean concentration of serum of all monkeys (B)

Table 1: Pharmacokinetic parameters of single dose injections

Parameter	Unit	APZ001			Cetuximab
		Group 1 ***7.5 mg/kg***	***Group 2*** ***25 mg/kg***	***Group 3*** ***75 mg/kg***	***Group 4*** ***25 mg/kg***
$t_{1/2}^{\#}$	h	26.29±4.6^{aa}	69.11±21.43	50.92±46.69	61.81±38.64
$t_{1/2}^{*}$	h	54.02±12.15^{aaa}	95.85±17.3^{b}	139.78±26.02^{ccc}	92.10±20.73
T_{max}	h	0.67±0.26	0.5±0.0	1.33±1.33	0.67±0.26
C_{max}	mg/mL	183.08±10.89^{aaa}	642.98±49.97^{bbb}	2132.31±229.53^{ccc}	617.28±65.42
$AUC_{(0-t)}$	h•μg/mL	9658.61±1251.54^{aaa}	53582.58±7824.48^{bbb}	181970.93±18381.55^{ccc}	45867.22±5770.93
$AUC_{(0-inf)}$	h•μg/mL	9661.09±1250.66^{aaa}	53608.67±7852.95^{bbb}	183350.05±20360.84^{ccc}	45873.39±5769.98
$AUC_{(t-inf)}$	%	0.03±0.02	0.04±0.06	0.66.±1.6	0.01±0.01
V_d	mL/kg	29.81±6.03	48.26±20.36	28.86±22.92	52.74±40.6
CL_s	mL/h/kg	0.79±0.10^{aa}	0.47±0.07	0.41±0.05^{ccc}	0.55±0.07
MRT	h	58.3±8.68^{aaa}	116.43±15.82	132.92±31.86^{ccc}	95.44±6.56

$^{\#}$Represents efficacy half-life, * represents terminal phase half-life. a, p < 0.05; aa, p < 0.01; and aaa, p < 0.001 compared with Group 2. b, p < 0.05; bb, p < 0.01; and bbb, p < 0.001 compared with Group 3. c, p < 0.05; cc, p < 0.01; and ccc, p < 0.001 compared with Group 1.

Table 2: IgA level during 6-month chronic toxicity test

Group	Concentration (μg/mLl)					
	D4, n=10	***W4, n=10***	***W13, n=9***	***W26, n=7***	***W31, n=3***	***W37, n=2***
Negative control	180.2±22.8	2.5.6±43.7	269.4±17.0 #	182.8±23.9	152.2±29.3 #	199.6±11.1
Cetuximab control	163.5±28.5	196.5±60.0	247.8±33.0 #	161.0±16.7	140.1±53.0	203.1±0.0 #
APZ001, low	162.3± 17.8	194.8±30.1#	252.5±20.7 #	172.4±27.9	210.8±21.5	193.1±58.6
APZ001, medium	187.9±50.1	204.1±24.7	256.9±29.6 #	168.8±20.6	191.1±16.4	223.1±26.3
APZ001, high	195.7±28.5	227.9±31.7	257.1±11.2 #	160.1±33.4	178.4±15.3	245.3±15.2

 # Represents p < 0.05 compared with blank serum before drug treatment. D, days; W, weeks.

Table 3: IgG level during 6-month chronic toxicity test

Group	Concentration (μg/mL)					
	D-4, n=10	W4, n=10	W13, n=9	W26, n=7	W31, n=3	W37, n=2
Negative control	12.6±2.0	14.0±3.7	17.0±1.2#	12.6±1.2	10.4±1.6#	13.7±0.3
Cetuximab	11.9±2.2	13.5±5.1#	16.1±2.0#	11.5±0.7	12.1±1.6	13.2±0.6
APZ001, low	11.3±0.8	14.2±2.6#	17.3±1.3#	11.7±1.2	12.8±0.3#	14.1±0.1#
APZ001, medium	11.6±1.4	14.3±1.6#	18.3±1.9#	11.6±0.8	11.7±0.4	11.2±0.3*
APZ001, high	12.8±2.0	15.5±3.5	16.9±0.8#	12.2±1.1	12.3±0.1	12.3±0.8

 # represents p < 0.05 compared with blank serum; represents p < 0.05 compared with negative control. D, days; W, weeks*

and APZ001 groups exhibited similar changes in levels of GCB, ALB, A/G, ALT, GGT, GLDH and AST, indicating the similar liver toxicity effects.

The levels of other kidney function markers, including urine nitrite, glucose, protein, bilirubin, urobilinogen, acetone bodies and white cell count, did not exhibit abnormality in all tested groups. The medium-dose APZ001 and cetuximab groups showed similar side effects (e.g. skin toxicities), which did not fully recover. Changes in pathology and lesions to other organs were not observed, nor were abnormalities in the cynomolgus monkeys' body weight, rectal temperature, blood pressure, hematological coefficients and coagulation function. In conclusion, after the 6-month chronic toxicity test, various drug administrations did not cause significant changes, which included the rectal temperature, hematological and coagulation function, electrocardiogram, blood pressure, urea biomarkers, pathological examination, bone marrow and optical examination.

Immunogenicity

Serum immunoglobins IgA, IgE, IgG, and IgM were semi-quantified by ELISA according to the manufacturer's instructions with slight modification. Quantification of serum IgA showed the results at eight time points were significantly different from the blank serum control (Weeks 4, 13, 31, and 37; $p < 0.05$; Table 2), but were the same as the positive control cetuximab group. During the week 13, a potential systematic error caused the results acquired from the blank group different from the blank serum. Quantification of serum IgG showed the results collected at eleven time points were different from the corresponding blank serum controls (both positive and negative) (Table 3, $p < 0.05$), indicating a systematic error. Several results obtained from the medium-dose cetuximab group showed differences from the negative control group (Table 2, $p < 0.05$). Quantification of of IgE and IgM showed that the results were not significantly different from the negative groups.

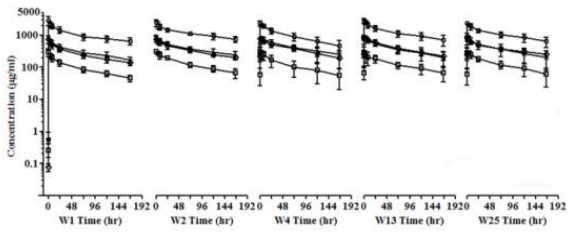

Figure 3: Pharmacokinetics of 6-month chronic drug administration. C_{max} and C_{min} changed in a dose-dependent manner. The T_{max}, T1/2, and other parameters did not change significantly ($p > 0.05$). There was no obvious change in all pharmacokinetic parameters upon comparing cetuximab and APZ001 (24 mg/kg).◊represents 25 mg/kg cetuximab; □represents 7.5 mg/kg APZ001;Δ represents 25 mg/kg APZ001;○ represents 75 mg/kg APZ001

DISCUSSION

In addition to internal organ epithelium cells, EGFR was also reported to be expressed in the human skin within keratinocytes, the follicular epithelium, sweat and sebaceous glands and

capillaries of the dermis, [11,12]. Disturbance of EGFR signaling might result in hair follicle necrosis and alopecia [13,14]. Minor side effects were reported in an earlier acute toxicity study, such as hair disorganization and partly alopecia in the cynomolgus monkeys [15]. Additionally, blocking EGFR function caused pathological reactions, such as skin inflammation, folliculitis and rash, due to the presence of EGFR in sebaceous glands [16].

In this work, APZ001 was well metabolized and did not accumulate upon weekly i.v. administration for 6 months. In direct comparison with cetuximab, weekly administration of APZ001 did not induce any unprecedented adverse effects, while the predictable anti-EGFR–related side effects, such as skin rash, dehydration and liquid feces, occurred at acceptable levels and rates.

Administration of cetuximab and APZ001 increased the kidney weight and organ coefficient, but no pathological changes or lesions were observed. No apparent damage was observed in the kidneys upon pathological examination, nor were any hematological markers found. Previous study using fluorescent dye-labelled cetuximab also indicated moderate and acceptable toxicity to organs [17]. In addition, similar results were also reported by using a mixture of two biosimilars of cetuximab in the cynomolgus monkeys [18].

Long-term drug administration led to the accumulation of drug in the circulation system, thus enhancing the kidney excretion activity to metabolize the excessive drug; the enhanced kidney function was maintained over a long course of time, leaving it impossible to recover in two recovery periods.

Slight to moderate skin toxicity side effects occurred to some cynomolgus monkeys. It was speculated that complete blocking of EGF-EGFR function in skin tissue might cause epidermal cell death occurring at a faster rate than cell growth, thus inducing skin toxicity. Many clinical studies of cetuximab have shown a direct correlation between the severity of rash and efficacy of treatment [19,20]. Harandi and colleagues reported that patients with grade 3 rash had the highest survival rate during the treatment of cetuximab [21]. Administrations of APZ001 and cetuximab (38 and 24 mg/kg doses, respectively) induced similar toxicity reactions in skin and eyes, indicating that the chronic toxicity of APZ001 was within the safe range for human use.

CONCLUSION

The biosimilar, APZ001, showed similar properties to the positive control, cetuximab, including its pharmacokinetics, toxicokinetics, acute toxicity, chronic toxicity and immunogenicity toxicity. Thus, APZ001 antibody may show similar therapeutic effect to cetuximab.

DECLARATIONS

Acknowledgement

This study was supported by the funds from the High-level Leading Talent Introduction Program of Guangdong Academic of Science, People's Republic of China (Number 2016GDASRC-0104 and 2016GDASRC-0206).

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaofei Wang wrote the manuscript. Wei Yang, Jianmin Guo and Huiqing Liang designed all the experiments and revised the manuscript. Xinyu Deng, Caiguo Ye, Yuankeng Huang and Xialing Lei performed the experiments.

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