European Journal of Pharmaceutical Sciences 109 (2017) 624–630

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Recombinant humanized anti-vascular endothelial growth factor monoclonal antibody efficiently suppresses laser-induced choroidal neovascularization in rhesus monkeys

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ARTICLE INFO

Chemical compounds studied in this article:
Sodium hydroxide (PubChem CID: 14,798)
Alamar blue (PubChem CID: 11,077)
Hematoxylin-eosin (PubChem CID: 86,598,188)
Pentobarbital sodium (PubChem CID: 23,676,152)
FITC (PubChem CID: 259,101)

Keywords:
VEGF
Monoclonal antibody
RabMAb
CNV
Wet AMD

ABSTRACT

Neovascular age-related macular degeneration, characterized by abnormal choroidal neovascularization (CNV), is a major cause of blindness worldwide. Anti-vascular endothelial growth factor (VEGF) antibodies have demonstrated significant efficacy in improving visual acuity. TMAB001 is a new recombinant humanized rabbit anti-VEGF monoclonal antibody. It presents high activities *in vitro* studies. In the binding affinity assay, TMAB001 exhibited a high binding capability to VEGF with an affinity constant of 10^{-11} M. In the receptor antagonist activity assay, IC_{50} of TMAB001 was 0.15 μg/ml. In a cell-based assay, TMAB001 inhibited $VEGF_{165}$-induced HUVEC cells proliferation in a dose-dependent manner. Furthermore, in the rhesus monkey model of laser-induced CNV, results showed the growth and leakage of experimental CNV were significantly decreased with a single bilateral intravitreal injection of TMAB001, and the grade 4 lesions were complete absence in TMAB001 groups. The efficacy of TMAB001 was maintained for at least 28 days. In a mice model of oxygen-induced retinopathy, the retina fluorescence leakage was reduced and the vascular morphology in retina was normalized by TMAB001 intraperitoneal administration. In conclusion, those results indicate that TMAB001 might be a potential drug candidate for wet AMD.

1. Introduction

Neovascular age-related macular degeneration (AMD) also known as wet AMD, characterized by abnormal choroidal neovascularization (CNV), is a leading cause of blindness around the world (Lim et al., 2012). Wet AMD occurs when abnormal blood vessels start to grow under the retinal pigment epithelium of the macula, originating a pathologic CNV whose severity ranges from small stable lesions with minimal visual impairment to large and rapidly growing lesions associated with hemorrhage, exudation, and neurodegeneration (Rezzola et al., 2014).

Since acute or sustained increases in intraocular pressure have been seen after intravitreal dosing with vascular endothelial growth factor inhibitors for the treatment of AMD (FDA, 2016), the volume of the intravitreal injection is 0.05ml in general. Due to the limited dosing volume, high affinity is necessary for topical ophthalmic drugs. Rabbit antibodies have been widely used in research and displayed therapeutic potential due to their high antigen specificity and affinity (Yano et al., 2016). Humanized anti-VEGF RabMAbs have been shown significantly inhibited the tumor growth of lung carcinoma and rhabdomyosarcoma xenografts in mice, as well as reduced the density of tumor microvessel in mice (Yu et al., 2010)(Yu et al., 2013). However, there were no related reports of the anti-VEGF RabMAbs on AMD. As anti-VEGF non-rabbit MAbs have demonstrated significant efficacy in improving visual acuity (VA) (Beck et al., 2016)(Heier et al., 2012), the anti-VEGF RabMAbs maybe also reveal enormous therapeutic potential.

TMAB001 (Jiangsu T-mab BioPharma Co., Ltd) is a full-length IgG1κ isotype humanized form of a rabbit MAb that selectively binds with high affinity to isoform of $VEGF_{165}$. The human framework contributes to 94% of the overall protein sequence. The molecular weight is 146 kDa.

TMAB001 is intended to be used in treatment of wet AMD and now

Abbreviations: AMD, age-related macular degeneration; CNV, choroidal neovascularization; VEGF, vascular endothelial growth factor; MAb, monoclonal antibody; VA, visual acuity; Kon, association rate; Kdis, dissociation rate; KDR, VEGF receptor-2; OD, optical density; IC_{50}, half maximal inhibitory concentration; FFA, fluorescein fundus angiography; OCT, optical coherence tomography; IVT, intravitreal; VEGFR, VEGF receptor

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http://dx.doi.org/10.1016/j.ejps.2017.09.021
Received 27 April 2017; Received in revised form 17 August 2017; Accepted 12 September 2017
Available online 13 September 2017
0928-0987/

under a phase I clinical trial (NCT02613559). The purpose of this preclinical study is to explore the activities and efficacy of TMAB001 *in vitro* and *in vivo* models related to wet AMD, in order to demonstrate the treatment potential of TMAB001 in wet AMD.

2. Materials and methods

2.1. Binding affinity of TMAB001 to $VEGF_{165}$

Bio-layer interferometry analysis was used to determine the binding affinity of TMAB001 to $VEGF_{165}$. NHS-Biotin (Thermo Fisher Scientific, USA) was conjugated to $VEGF_{165}$ (Epitomics, China) with a molar ratio of 1:10 as the instructions of NHS-Biotin reagents. Then the biotinylated $VEGF_{165}$ was loaded to a streptavidin (SA) sensor (Fortebio, USA) for 15 min at 200 rpm. After the loading process, the SA sensors were moved into the sample wells (TMAB001 and Avastin [Roche, Switzerland] of different concentrations; 30 min, 1000 rpm) to detect the association rate (Kon) of TMAB001 to $VEGF_{165}$. Then the above SA sensors were transferred into PBS solution wells (60 min, 1000 rpm) to detect the dissociation rate (Kdis). Affinity constant K_D value (Kdis/Kon) was calculated with Octet-QK system (Fortebio, USA).

2.2. Receptor antagonist activity to VEGF receptor-2

ELISA assay was used to evaluate the receptor antagonist activity of TMAB001. VEGF receptor-2 (KDR) was coated on ELISA plate at 4 °C overnight. A certain amount of VEGF was pre-incubated with serial concentrations of TMAB001 at 37 °C for 1 h. Following washing the KDR-coated plate with TBST, the VEGF-TMAB001 complex was added into the plate and incubated at 37 °C for 1 h. After washing the unbound complex, a mouse anti-VEGF monoclonal antibody was used to detect the VEGF binding with the KDR, followed by incubation with goat anti-mouse secondary antibodies labeled with alkaline phosphatase. The chromogenic reaction started after the substrate was added and was terminated after NaOH was added. The optical density (OD) value of each well at wavelength 405 nm was obtained using the Microplate Reader (Spectra Max M5, Molecular Devices, USA), then the IC_{50} of TMAB001 was calculated from the dose-response curves using the SoftMax Pro 5.4.1 software.

2.3. Inhibition of $VEGF_{165}$-induced proliferation of human umbilical vein endothelial cells (HUVEC)

Primary HUVEC (Sciencell, USA) were cultured in a conditioned medium containing 320 ng/ml $VEGF_{165}$ and a serial concentrations of TMAB001 for 72 h, then 10% Alamar blue fluorescent dye (Thermo Fisher Scientific, USA) was added into each well. OD value at wavelength 570 nm with 600 nm for reference was obtained using the Microplate Reader after 24 h. The IC_{50} of TMAB001 was calculated from the dose-response curves using the SoftMax Pro 5.4.1 software.

2.4. Inhibitory effect on the monkey model of CNV

The effect of TMAB001 treatment on laser-induced CNV was evaluated in rhesus monkeys (Sichuan Greenhouse Biotech, Chengdu, China). All of the experimental methods and techniques were in accordance with the AAALAC International Standard. All procedures performed in studies involving monkeys were in accordance with the ethical standards of the institution at which the studies were conducted.

CNV was induced with a laser (Vissulus 532s Laser Photo-coagulator Carl Zeiss Meditec AG, Jena, Germany) through a + 78 D preset lens. Laser photocoagulation was conducted around the fovea but avoided damage to the fovea and 6 to 8 lesion points were induced in each eye. Laser lesions were placed in a circular fashion around the macula. The development of CNV lesions was assessed by fluorescein fundus angiography (FFA) and optical coherence tomography (OCT) once before injury and 20 days to 21 days after laser injury.

Table 1
Kinetic parameters of binding affinity.

Samples	KD (M)	Kon (1/Ms)	Kdis (1/s)	Full X^2	Full R^2
TMAB001	3.295E-11	1.471E + 05	4.846E-06	0.310962	0.991249
Avastin	1.236E-10	5.046E + 04	6.239E-06	0.546444	0.996646

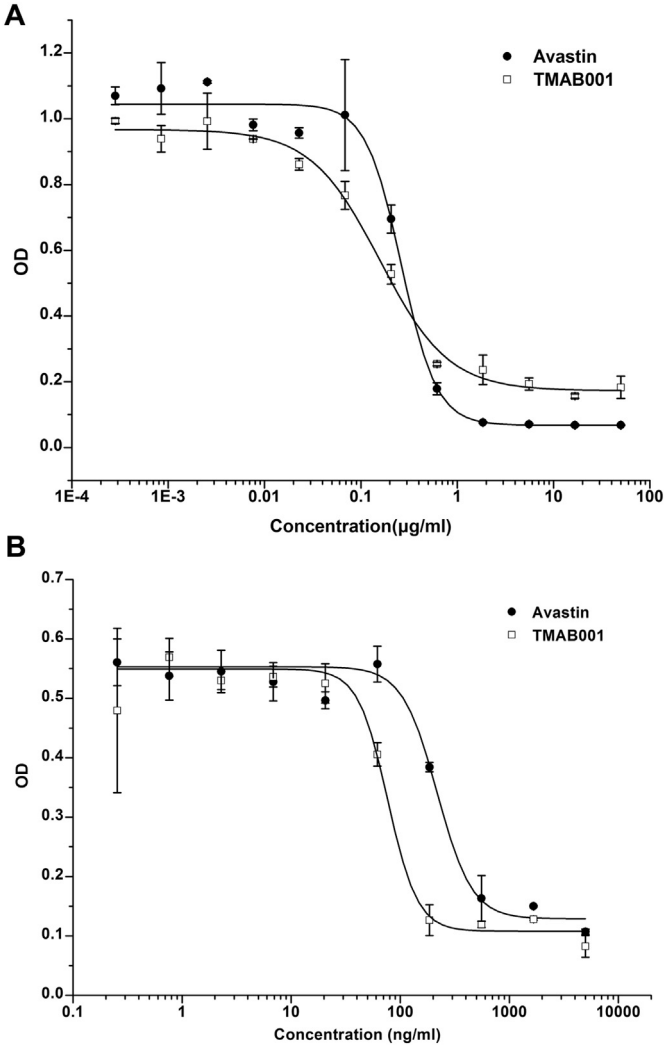

Fig. 1. TMAB001 exhibited high antagonizing activity to KDR (A) and inhibiting effect on the $VEGF_{165}$-induced HUVEC proliferation (B). A. Receptor antagonist activity of TMAB001 and Avastin to KDR. The mean IC_{50} of Avastin was about 1.72 times of that of TMAB001. B. Inhibition of the $VEGF_{165}$-induced HUVEC proliferation. IC_{50} value of Avastin was about 2.8 times of that of TMAB001.

Twenty-one days after the laser applications, 20 monkeys were chosen based on their fluorescein fundus angiography (FFA) and optical coherence tomography (OCT) detection and were divided into five groups to receive bilateral intravitreal (IVT) of 50 μl/eye balanced salt solution (BSS; Alcon Canada.), Avastin 1.25 mg/eye, TMAB001 0.625, 1.25 and 2.5 mg/eye, respectively. Fundus color photography and FFA were performed with a fundus camera (TRC-50DX, Topcon, Japan) on the day before laser induction, the day of laser induction (FFA only) and 20 days after laser induction, 14 and 28 days after drugs injection. Color fundus photography and FFA were used to detect and measure the extent and evidence of angiographic leakage and/or any other abnormalities. Angiographically, the burn is hypofluorescent early. If CNV is present, hyperfluorescence develops around the burn, which