

Supplemental Information

Tetravalent Bispecific Tandem Antibodies

Improve Brain Exposure and Efficacy

in an Amyloid Transgenic Mouse Model

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Table S1. Pharmacokinetic data of TBTI1-6 in WT mice

Dosing	Strain	Compound	Matrix	C _{max} or C ₀	t _{max}	AUC _{0-3day}	AUC _{0-7day}	t _{last}	Max % of injected dose / g of brain	Brain / Plasma AUC _{0-3day} ratio%	Brain / Plasma AUC _{0-7day} ratio%	TBTi / 13C3 Brain max%	TBTi / 13C3 Brain AUC	Study	RA / Batch
				(nmol/L or /kg)	(day)	(nmol.day/L or /kg)	(nmol.day/L or /kg)	(day)		ratio	ratio	ratio	ratio	Ref.	Id.
IV Bolus 70 nmol/kg	Wild type	13C3	Plasma	899	-	1420	2770	7	-	-	-	-	-	ABS0758	SAR220590
			Total Brain	0.445	3	0.827	2.64	7	0.0337	0.058	0.0954	-	-		
			Parenchyma	-	-	-	-	-	-	-	-	-	-		
		13C3	Plasma	1260	-	1660	3390	7	-	-	-	-	-	ABS0721-2	SAR220590
			Total Brain	-	-	-	-	-	-	-	-	-	-		
			Parenchyma	0.589	[1 - 7]	1.24	3.36	7	0.036	0.075	0.099	-	-		
		TBTi 1 TBTi-(anti-Abeta-13C3 x anti-TfR-8D3)-mulgG1	Plasma	NC	-	434	436	7	-	-	-	-	-	ABS0758	RA10876562
			Total Brain	4.42	1	8.88	10.1	3	0.298	2.05	2.32	8.84	10.7		
			Parenchyma	2.04	1	4.34	5.28	7	0.14	1.00	1.21	3.9	3.5		
		TBTi 1 TBTi-(anti-Abeta-13C3 x anti-TfR-8D3)-mulgG1	Plasma	1390	-	513	-	3	-	-	-	-	-	ABS0721-4	RA10876562
			-	-	-	-	-	-	-	-	-	-	-		
			Parenchyma	2.27	[0.083-1]	5.18	-	3	0.13	1.00	-	3.6	4.2		
		TBTi 2 TBTi-(anti-TfR-8D3 x anti-Abeta-13C3)-mulgG1	Plasma	1090	-	357	-	3	-	-	-	-	-	BPK-VA-50006	VAB.QDJ3.89B
			Total Brain (TfR capture)	4.12	0.083	7.99	-	3	0.28	2.24	-	8.2	9.7		
			Parenchyma	-	-	-	-	-	-	-	-	-	-		
		TBTi 3 TBTi-(anti-Abeta-13C3xanti-TfR-8D3)-LCY92A-mulgG1	Plasma	NC	-	493	-	3	-	-	-	-	-	ABS0721-14a	VA216205
			Total Brain	5.08	1	12.1	-	3	0.322	2.45	-	9.55	14.6		
			Parenchyma	3	1	6.88	-	3	0.19	1.40	-	5.3	5.5		
		TBTi 4 TBTi-(anti-Abeta-13C3xanti-TfR-8D3)-HCY52A-mulgG1	Plasma	1450	-	1600	-	3	-	-	-	-	-	ABS0721-14b	VA216206
			Total Brain	2.61	1	6.93	-	3	0.116	0.433	-	3.44	8.38		
			Parenchyma	1.79	1	4.86	-	3	0.08	0.304	-	2.2	3.9		
		TBTi 5 TBTi-(anti-Abeta-13C3xanti-TfR-8D3)-HCY52A-LCY92A-mulgG1	Plasma	1410	-	1770	-	3	-	-	-	-	-	BPK-VA-50007	VAB.QDJ3.88B
			Total Brain	1.44	3	3.65	-	3	0.0921	0.21	-	2.73	4.41		
			Parenchyma	-	-	-	-	-	-	-	-	-	-		
		TBTi 6 Anti-Abeta-13C3xanti-TfR-8D3 HC-Y52A-S101A LC-Y92A-Y49A mulgG1	Plasma	1230	-	1840	-	3	-	-	-	-	-	BPK-VA-50011	VA2-16-326-1
			Total Brain	3.61	1	8.06	-	3	0.216	0.439	-	6.41	9.75		
			Parenchyma	2.91	1	6.79	-	3	0.174	0.368	-	4.8	5.5		

Anti-TfR/anti-A β (TBTi) or control antibody was administered to wild-type (WT) mice at a dose of 70 nmol/kg. Blood samples were collected in heparinized tubes from posterior vena cava at 5 min and at 2, 5, 24 and 72 h after single IV bolus administration and plasma collected by centrifugation for 10 min at 1500 g. Immediately after blood sampling, mice were perfused via the left ventricle with 50 mL of ice-cold phosphate buffer saline (524560, Calbiochem, San Diego, CA). The mouse brain cortex was harvested, frozen immediately on dry-ice and stored at -80°C until use for antibody quantification. Pharmacokinetic analyses were performed using non-compartmental method (Phoenix-WinNonLin, version 6.4).

Table S2. Plasma parameters (nmol/L or /kg) of 13C3 and TBTI1 concentrations (nmol/L or /kg) following intravenous (70 nmol/kg) administration to C57BL/6 Wild type and APP/PS1 transgenic male mice

Compound	Mouse strain	C0 or Cmax (nmol/L)	AUC0-7day (nmol.day/L)
13C3	WT	899	2770
	APP/PS1	959	3550
TBTI1	WT	569	436
	APP/PS1	766	807

Anti-TfR/anti-A β (TBTI1) or control antibody 13C3 were administered to wild-type (WT) or APP/PS1 mice at a dose of 70 nmol/kg. Blood samples were collected in heparinized tubes from posterior vena cava at 5 min and at 2, 5, 24 and 72 h after single IV bolus administration and plasma collected by centrifugation for 10 min at 1500 g. Pharmacokinetic analyses were performed using non-compartmental method (Phoenix-WinNonLin, version 6.4).

Table S3. Hematological evaluation of APPSL mice treated with anti-A β (TBTIs or 13C3) or control IgG1 antibodies

Groups		RBC	HGB	HCT	MCV	MCH	MCHC	RDW	Platelet	Reticulocytes	WBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	LU Cells
		10E12/L	g/L	%	fl	pg	g/L	%	10E9/L	10E12/L	10E9/L	10E9/L	10E9/L	10E9/L	10E9/L	10E9/L	10E9/L
Ctrl IgG1 (70 nmol/kg)	Mean	9.070	132.3	45.67	50.37	14.57	289.0	13.3	1219	0.254	4.14	0.567	3.303	0.147	0.113	0.003	0.015
APPSL	STD	0.267	4.041	1.557	0.416	0.208	2.646	0.361	100.409	0.017	1.77	0.247	1.745	0.146	0.040	0.006	0.021
13C3a (70 nmol/kg)	Mean	10.031	142.6	49.23	49.07	14.21	289.6	13.24	1110.3	0.2514	5.234	0.531	4.501	0.080	0.097	0.007	0.030
APPSL	STD	0.305	4.6	1.31	0.75	0.24	4.1	0.25	204.6	0.0111	0.900	0.243	0.892	0.051	0.078	0.005	0.012
TBTI3a (15 nmol/kg)	Mean	9.083	124.0	43.68	48.10	13.63	283.8	15.78	1129.0	0.4355	4.553	0.742	3.540	0.105	0.143	0.008	0.020
APPSL	STD	0.358	5.8	2.14	1.32	0.44	7.0	0.84	163.1	0.1184	0.682	0.623	0.352	0.113	0.045	0.004	0.007
TBTI6a (15 nmol/kg)	Mean	9.656	139.0	47.90	49.63	14.40	290.4	13.15	1197.6	0.2516	4.525	0.411	3.930	0.064	0.098	0.006	0.019
APPSL	STD	0.345	5.3	1.68	0.84	0.20	3.9	0.39	112.2	0.0253	0.710	0.189	0.669	0.085	0.051	0.005	0.009
Ctrl IgG1 (70 nmol/kg)	Mean	9.585	143.0	48.10	50.35	14.95	297.0	13.00	1040.5	0.2895	7.170	0.870	6.005	0.140	0.100	0.015	0.045
WT	STD	0.629	2.8	0.71	4.03	1.34	1.4	0.14	163.3	0.0502	0.778	0.523	0.361	0.028	0.057	0.007	0.021

Male APPSL mice were treated once a week by IP administration with one of the following antibodies: Ctrl-IgG1 (70 nmol/kg), 13C3a (70 nmol/kg), TBTI3a (15 nmol/kg) and TBTI6a (15 nmol/kg) for 4 months. WT control mice were also treated with 70 nmol/kg of Ctrl-IgG1 under the same regimen. One week after the last treatment, animals were sacrificed, and blood was collected for hematological studies. Compared to control IgG1 treated groups, slightly to moderately lower RBC mass parameters and moderately higher reticulocytes and neutrophils counts were observed for mice treated with TBTI3a at 15 nmol/kg. No difference in hematological parameters was observed for TBTI6a treated group.

Table S4. Histopathological evaluation of APPSL mice treated with anti-A β (TBTIs or 13C3) or control IgG1 antibodies

Treatment groups	Animal number	Liver histopathology	Spleen histopathology
APPSL Ctrl IgG1 70 nmol/kg	12	Minimal necrosis	Minimal extramedullary hemopoiesis
	18	Within normal limits	Minimal decreased white pulp cellularity
	21	Within normal limits	Within normal limits
	35	Minimal necrosis	Within normal limits
	39	Minimal necrosis	Minimal increased white pulp cellularity
	43	Within normal limits	Within normal limits
	45	Mild centrilobular hypertrophy	Within normal limits
	47	Within normal limits	Within normal limits
	51	Within normal limits	Within normal limits
APPSL TBTI3a 15 nmol/kg	19	Minimal centrilobular hypertrophy	Mild extramedullary hemopoiesis
	26	Minimal centrilobular hypertrophy Minimal necrosis	Minimal extramedullary hemopoiesis
	29	Minimal centrilobular hypertrophy	Mild Extramedullary hemopoiesis
	30	Mild centrilobular hypertrophy Mild mononuclear cell infiltration	Minimal decreased white pulp cellularity
	33	Minimal necrosis	Minimal extramedullary hemopoiesis
	46	Within normal limits	Within normal limits
	49	Within normal limits	Within normal limits
	50	Minimal centrilobular hypertrophy Minimal necrosis	Minimal extramedullary hemopoiesis
	13	Minimal mononuclear cell infiltration	Within normal limits
APPSL 13C3a 70 nmol/kg	17	Minimal necrosis	Within normal limits
	24	Within normal limits	Minimal decreased white pulp cellularity
	25	Within normal limits	Within normal limits
	32	Minimal necrosis	Minimal decreased white pulp cellularity
	34	Minimal necrosis	Within normal limits
	38	Within normal limits	Minimal decreased white pulp cellularity
	41	Within normal limits	Within normal limits
	44	Within normal limits	Within normal limits
	14	Minimal necrosis	Within normal limits
	15	Mild necrosis	Within normal limits
APPSL	16	Minimal necrosis	Minimal decreased white pulp cellularity
	20	Minimal mononuclear cell infiltration	Minimal decreased white pulp cellularity

TBTI6a 15 nmol/kg	22	Within normal limits	Minimal decreased white pulp cellularity
	27	Within normal limits	Within normal limits
	31	Within normal limits	Minimal decreased white pulp cellularity
	37	Minimal necrosis	Minimal decreased white pulp cellularity
	52	Minimal necrosis	Within normal limits
	53	Within normal limits	Within normal limits
WT Ctrl IgG1 70 nmol/kg	1	Minimal centrilobular hypertrophy	Minimal increased white pulp cellularity
	2	Minimal mononuclear cell infiltration	Minimal decreased white pulp cellularity
	3	Minimal necrosis	Within normal limits
	4	Within normal limits	Within normal limits
	5	Minimal centrilobular hypertrophy	Within normal limits
	6	Within normal limits	Within normal limits
	7	Minimal necrosis	Within normal limits
	8	Mild hepatocellular degeneration with increased number of mitotic figures, abnormal mitosis and anisokaryosis.	Within normal limits
	9	Minimal necrosis	Within normal limits
	10	Within normal limits	Within normal limits
	11	Within normal limits	Within normal limits

Male APPSL mice were treated once a week by IP administration with one of the following antibodies: Ctrl-IgG1 (70 nmol/kg), 13C3a (70 nmol/kg), TBTI3a (15 nmol/kg) and TBTI6a (15 nmol/kg) for 4 months. WT control mice were also treated with 70 nmol/kg of Ctrl-IgG1 under the same regimen. One week after the last treatment, animals were sacrificed, and liver and spleen were harvested and treated with 4% formaldehyde for histopathological studies.

Table S5. Comparison of TBTI3 after 1 and 2 administrations

AUC0-3d (nmol.d/L or /kg)		B/P	Max %ID/g	Dose	Administration	
Brain	Plasma					
12.1	493	2.44	0.322	14 mpk (70 nmolKg)	IV	Single dose
5.00	56.9	8.78	0.783	4 mpk (20 nmolKg)	IP	Repeat dose

Anti-TfR/anti-A β (TBTI3) was administered to wild-type (WT) mice at a dose of 70 nmol/kg IV and 20 nmol/kg IP, respectively. Blood samples were collected in heparinized tubes from posterior vena cava at 5, 24 and 72 h after single IV bolus and 2 repeat IP administrations and plasma collected by centrifugation for 10 min at 1500 g. Immediately after blood sampling, mice were perfused via the left ventricle with 50 mL of ice-cold phosphate buffer saline (524560, Calbiochem, San Diego, CA). The mouse brain cortex was harvested, frozen immediately on dry-ice and stored at -80°C until use for antibody quantification. Pharmacokinetic analyses were performed using non-compartmental method (Phoenix-WinNonLin, version 6.4).

Figure S1. Kinetic curves of Total Radiance Efficiency detected in blood samples of wild-type as compared to transgenic APPSL mice, following caudal IV administration of the labeled fluorescent antibody TBTI3a-AF750. The fluorescent molecules were detected by *ex vivo* epi-illumination imaging procedures (at emission filter: 800 nm; excitation filter: 745 nm) applied on blood samples collected in a well-plate: at 3; 72 and 168 h following TBTI3a-AF750 intravenous administration. Each symbol represents the mean (\pm the SEM) of detected total radiance efficiency (flux).

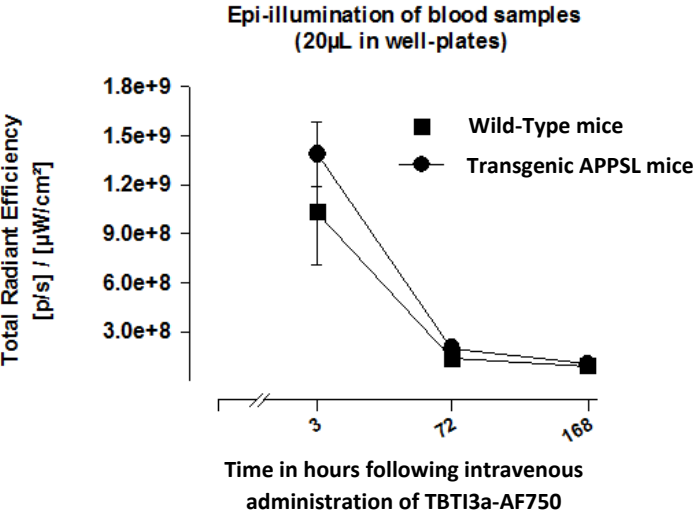


Figure S2. Illustration of TBTI3-AF488 labelled antibody detectable in vasculature APPSL transgenic mouse. Representative images of green fluorescent of TBTI3-AF488 labelled administered antibodies (72 h after second IV injections at 20 mg/kg) within thalamic, cortical and hippocampal vascular structures. Tissue was counterstained with DAPI. Scale bar equals 50 μ m.

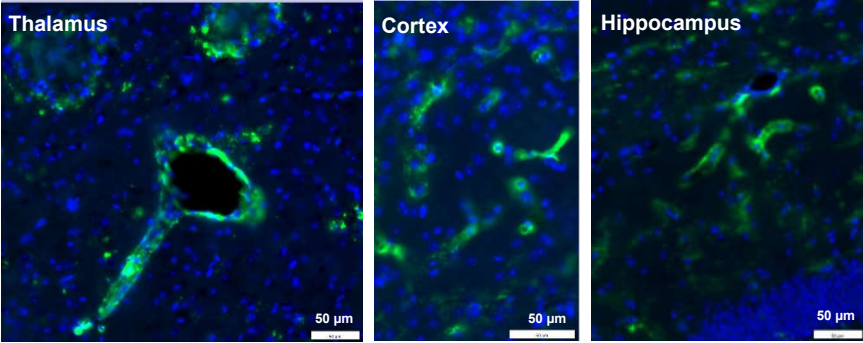


Figure S3: Unexplained increase of small A β deposit number in hippocampus of APPSL transgenic mice treated for 4 months with TBTI3a antibody. Representative images of A β immunolabelling in hippocampal parenchyma of 4 animals treated with control Ctrl-IgG1 (A, B, E, F) and 4 animals treated with TBTI3a antibody (C, D, G, H).

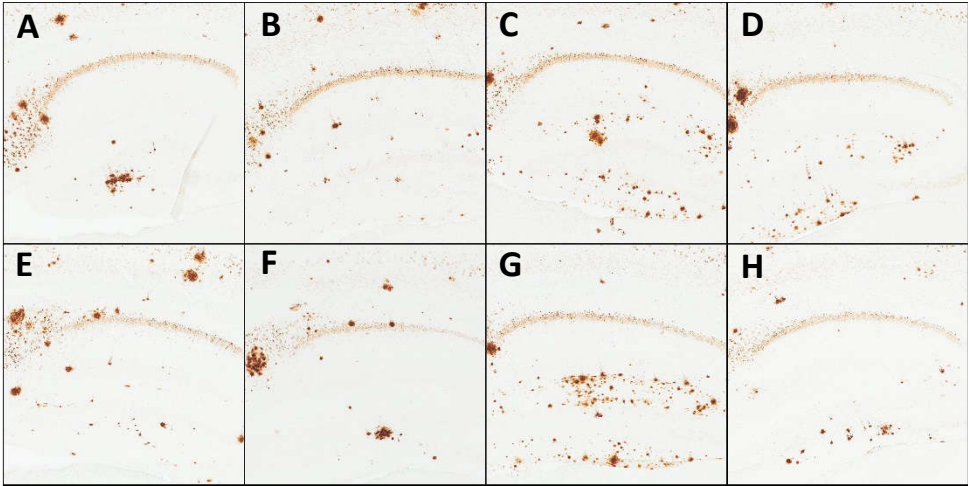


Figure S4: *In vitro* uptake of anti-TfR/anti-A β antibodies TBTI2a, TBTI3a and TBTI6a in mouse primary neurons and in the mouse brain microvascular endothelial bEnd.3 cell line. (A) Antibody uptake in mouse primary cortical neurons (DIV10) and (B) in bEnd.3 cells following an exposure to each of the tested constructs at 5 nM and 50 nM, respectively, for 2 h at 37°C. Following the incubation period, cells were rinsed with PBS (-/-) containing 0.5% acetic acid, fixed with PFA 4%, permeabilized in PBS 0.2% Triton X-100, and probed for the internalized antibody with either AF633-coupled or AF488-coupled anti-mouse IgG. Cellular membranes and nuclei were stained by using Wheat Germ Agglutinin (WGA) coupled to AF555 and Hoescht33342, respectively. Confocal images were obtained using an Operetta CLS™ high-content analysis system.

