Electronic Supplementary Material

Clinical translation of [^{68}Ga]NOTA-anti-MMR-sdAb for PET/CT imaging of protumorigenic macrophages

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Supplemental Materials and Methods:

Dosimetry Studies

Mouse biodistribution data was obtained via dissection and gamma counting at 10, 60, 120 and 180 min post injection for the following organs or tissues: adrenals, brain, thymus, thyroid, blood, heart (wall), lungs, liver. Uptake of both kidneys was summed for further calculations. For each organ and time point, the uptake was calculated as mean±SD, expressed as %IA and %IA/g. The following organs were used as source organs in OLINDA/EXM version 1 software: adrenals, brain, thymus, thyroid, heart, lungs, kidneys, spleen, stomach, large intestine, upper and lower large intestine (each as 50% of total large intestine uptake value), gallbladder. All activity not present in those organs was attributed to the remainder of the whole body (excluding any bladder activity). For each source organ, bi-exponential fit was performed within the software, and the total disintegrations per source organ were estimated based on this bi-exponential fit. To account for any disintegrations occurring within the urinary bladder, a voiding bladder model was applied with a voiding interval of 1h, a renal excretion of 100% and using the calculated biological half-life. The biological half-life was calculated specifically for this voiding bladder model within OLINDA/EXM version 1 software; it was based the whole body activity over time (excluding any urinary activity in the urinary bladder) in dissected mice and by applying a mono-exponential fit. The whole body activity was calculated based on the counts in the organs, measured in a gamma counter and on the counts in the dissected mice, measured in the ionization chamber, at different time points.

Based on the adult models available within the software and accounting for cross-irradiation of source organs to all organs contributing to the effective dose or effective dose equivalent, the organ and total body doses are calculated. Doses are calculated using the appropriate weighting factors for the various organs.

Toxicity Study with NOTA-anti-MMR-sdAb

A 7-day intravenous repeated dose toxicity study of the NOTA-anti-MMR-sdAb was performed in compliance with Good Laboratory Practice (GLP) Regulations, as required by regulatory

authorities for subsequent human use (Eurofins BioPharma Product testing, Germany). The ICH M3 (R2) guideline was applied *(EMA/CPMP/ICH/286/95))*.

The objective of the study was to assess the possible health hazards, which could arise from repeated exposure of NOTA-anti-MMR-sdAb via intravenous administration to mice over a period of 7 days. The test item (at 1.68 mg/kg body weight) or control item (PBS) were administered daily intravenously to 20 male and 20 female Balb/c mice (7-8-week-old) for a period of 7 days. During the period of administration, the animals were observed daily for signs of toxicity. One day after the last administration, blood was sampled for hematology and clinical biochemistry and a complete necropsy was performed in 10 animals per group and gender. Blood samples were analyzed for levels of hematocrit, hemoglobin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatinine, total protein, albumin, urea, total bilirubin, glucose, sodium, potassium, calcium, chloride and inorganic phosphate, and number of red blood cells, reticulocytes, platelets, white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils and large unstained cells. Urine analysis was performed at the end of the experiment. The following parameters were analyzed: specific gravity, pH, levels of nitrite, protein, glucose, ketone bodies, urobilinogen, bilirubin, and numbers of erythrocytes and leukocytes. The organ weight of a defined set of organs was measured. Histopathological examination was performed for 40 selected organs. In order to allow detection of possible delayed occurrence or persistence of or recovery from toxic effects, 10 recovery animals per group and gender were observed for a period of 14 days following the last administration and were subjected to necropsy thereafter. Body weight and food consumption were measured weekly.

In addition to classical toxicity testing, also cytokine levels in the blood were determined in 5 satellite animals per group and per gender at 2h and 6h after single administration. Cytokine testing was performed in conformity with internal quality assurance regulations, on the basis of GLP regulations, but was not audited and therefore it does not have a GLP status. Cytokine determination was performed according to the manufacturer's instructions of the kit (V-PLEX Plus Proinflammatory Panel 1 (mouse) Kit, Cat. No. K15048G-1) and included the determination of the following mouse cytokines: IFN-γ, IL-1ß, IL-10, IL- 12p70, IL-6, KC/GRO and TNF-α.

Supplemental Table 1. Stability data of NOTA-anti-MMR-sdAb at -20°C and accelerated stability testing at 4°C.

Release Test	***Real-Time Time point testing (-20 °C)***			***Accelerated stability testing (4 °C)***	
	T0	**T3m**	**T6m**	**T3d**	**T7d**
Protein concentration	1.2mg/ml	1.2mg/ml	1.2mg/ml	1.2mg/ml	1.2mg/ml
Purity and Profile by SDS-PAGE and Coomassie staining	One major band at approx. 15 kDa 100%	One major band at approx. 15 kDa >99%	One major band at approx. 15 kDa >99%	One major band at approx. 15 kDa >99%	One major band at approx. 15 kDa >99%
Profile by SDS-PAGE and Western blotting	One major band at approx. 15kDa	NA	NA	NA	NA
pH	7	7	7	7	7
0.22 µm membrane filter integrity	3.6 bar	NA	NA	NA	NA
Endotoxin content (LAL test)	< 1EU/ml	NA	NA	NA	NA
Characterization Test	***Real-Time Time point testing (-20 °C)***			***Accelerated stability testing (4 °C)***	
	T0	**T3m**	**T6m**	**T3d**	**T7d**
Receptor affinity by SPR (nM)	TC: 1 RC1: 0.86 RC2: 0.72	NA	NA	NA	NA
Number of NOTA (ESI-Q-TOF-MS)	12660, 12677 (0) 13111, 13128 (1)	NA	NA	NA	NA
Sterility	ok	NA		NA	NA
Protein integrity by Analytic SEC	100%	99.7%	98.8%	99.3%	99.7%
^{68}Ga labeling (RCP by SEC and iTLC)	>99%	>99%	>98%	>99%	>99%

NA: not analyzed, TC: test compound; RC1: reference compound 1 (starting material for coupling); RC2: reference compound 2 (non-GMP his-tagged compound);

Supplemental Table 2. Three validation batches of NOTA-coupling procedure for NOTA-anti-MMR-sdAb..

Release Test	**Batch 1 30157/020517**	**Batch 2 30157/030517**	**Batch 3 30157/040517**
Protein concentration	0.96mg/ml	0.96mg/ml	1mg/ml
Profile and Purity by SDS-PAGE and Coomassie staining	One major band at approx. 15kDa >95 %	One major band at approx. 15kDa >95 %	One major band at approx. 15kDa >95 %
Profile by SDS-PAGE and Western blotting	One major band at approx. 15 kDa	One major band at approx. 15 kDa	One major band at approx. 15 kDa
pH	7	7	7
0.22 µm membrane filter integrity	4.2	4.2	4.2
Endotoxin content	2.4 EU/ml	1.7 EU/ml	2.48 EU/ml
Characterization Test			
Receptor affinity of SPR (nM)	TC: 1.3 RC1: 1.3 RC2: 1.2	TC: 1.2 RC1: 1.3 RC2: 1.2	TC: 1.4 RC1: 1.3 RC2: 1.2
Addition of NOTA (ESI-Q-TOF)	12661, 12677 (0) 13173, 13190 (1)□	12661, 12677 (0)⋆ 13173, 13190 (1)	12661, 12677 (0)⋆ 13173, 13190 (1)
Sterility	ok	ok	ok
Protein integrity by Analytic SEC	99 %	99 %	99 %

RC1: reference compound 1 (starting material for coupling); RC2: reference compound 2 (non-GMP his-tagged compound);

Supplemental Table 3. Characterization of ^{69,71}Ga-NOTA-anti-MMR-sdAb.

Characterization test	**Result**
Receptor affinity of SPR	1.3 nM 1.3 nM (anti-MMR- sdAb) 1.2 nM (anti-MMR- sdAb His6)
Addition of ^{69,71}Ga (ESI-Q-TOF-MS)	12660, 12677 (0) 13178, 13194 (1)
Protein integrity by Analytic SEC	99 %□
Purity by SDS-PAGE and Coomassie staining	>95 %
Profile by SDS-PAGE and CBB staining	One major band at approx. 15 kDa
Profile by SDS-PAGE and Western blotting	One major band at approx. 15 kDa

Supplemental Table 4. Biodistribution data in wild type (WT) and MMR knock-out (MMR-KO) mice bearing 3LL-R tumor, 3 h after intravenous injection of [^{68}Ga]Ga-NOTA-anti-MMR-sdAb. Dose of protein (NOTA-anti-MMR-sdAb) injected was 5 μg.

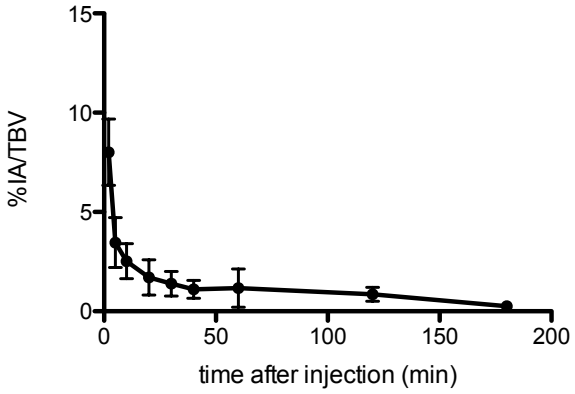
Tissue/organ	Uptake (% IA/g ± SD)		Uptake (% IA ± SD)	
	WT (n=5)	MMR-KO (n=5)	WT (n=5)	MMR-KO (n=5)
Lungs	0.96±0.16	0.21±0.06	0.16±0.02	0.03±0.01
Heart	0.95±0.19	0.10±0.01	0.10±0.01	0.01±0.00
Liver	3.64±0.62	0.43±0.08	3.78±0.76	0.43±0.07
Gall bladder	-	-	0.012±0.003	0.004±0.001
Kidney				
Left	73.77±9.70	96.35±20.89	9.42±0.31	10.36±1.13
Right	78.13±10.45	96.57±18.44	10.21±0.90	11.19±1.20
Stomach (+ content)	0.57±0.11	0.23±0.17	0.22±0.02	0.09±0.06
Small intestine (+ content)	0.58±0.10	0.25±0.10	0.59±0.12	0.27±0.07
Large intestine (+ content)	1.49±0.25	0.48±0.09	1.12±0.15	0.41±0.10
Spleen	2.17±0.54	0.36±0.12	0.28±0.09	0.04±0.01
Adrenals	-	-	0.03±0.01	0.02±0.00
Muscle	0.43±0.10	0.09±0.03	0.05±0.01	0.01±0.01
Bone	0.97±0.13	0.17±0.05	0.08±0.02	0.01±0.00
Lymph nodes	1.93±0.22	0.37±0.18	0.06±0.01	0.01±0.00
Blood	0.32±0.04	0.20±0.03	0.05±0.01	0.02±0.01
Tumor	2.24±0.28	0.40±0.14	1.43±0.39	0.28±0.08
Total %IA GI tract (stomach + intestines)	-	-	1.94±0.24	0.84±0.22

%IA: % of injected activity; GI: gastrointestinal

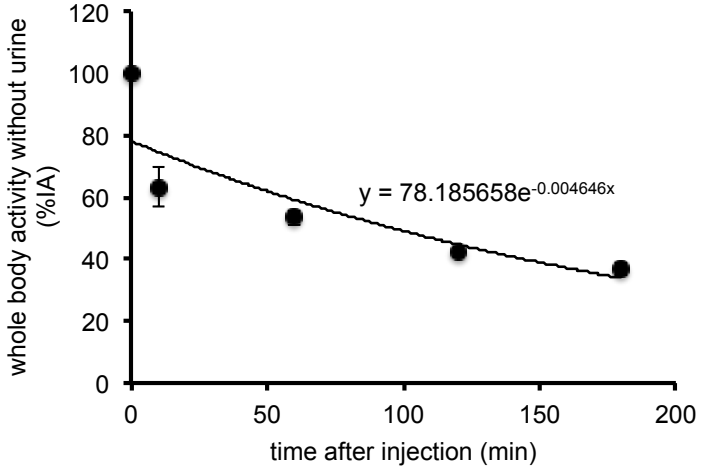
Supplemental Table 5. Biodistribution data for [^{68}Ga]Ga-NOTA-anti-MMR-sdAb at different time points for dosimetric analysis. Data is expressed as mean %IA ± SD (n=6), dose of protein (NOTA-anti-MMR-sdAb) injected was 5 μg

Tissue/organ	[^{68}Ga]Ga-NOTA-anti-MMR-sdAb uptake (%IA±SD)			
	10 min	60 min	120 min	180 min
Adrenals	0.02±0.01	0.010±0.005	0.008±0.002	0.009±0.002
Brain	0.03±0.00	0.012±0.003	0.011±0.002	0.008±0.003
Thymus	0.07±0.01	0.057±0.014	0.049±0.023	0.040±0.011
Thyroid	0.01±0.00	0.005±0.002	0.005±0.002	0.005±0.002
Blood	0.46±0.17	0.16±0.03	0.050±0.014	0.062±0.010
Heart	0.35±0.03	0.211±0.008	0.147±0.019	0.116±0.062
Lungs	0.42±0.04	0.252±0.037	0.200±0.021	0.190±0.016
Liver	13.92±2.37	8.751±1.993	5.307±0.581	3.831±0.292
Kidney Left	8.29±1.18	9.844±0.730	8.681±1.237	7.826±0.661
Kidney Right	8.70±1.11	10.635±0.316	8.529±0.942	8.703±0.623
Spleen	0.93±0.04	0.406±0.097	0.237±0.027	0.190±0.023
Stomach	0.66±0.07	0.461±0.212	0.319±0.039	0.284±0.045
Large Intestine	1.42±0.10	0.994±0.098	0.924±0.250	1.078±0.361
Small intestine	1.80±0.09	0.962±0.126	0.817±0.206	0.882±0.397
Gallbladder	0.04±0.02	0.051±0.056	0.020±0.007	0.012±0.004
Urinary bladder	9.91±3.63	1.792±1.167	5.326±1.773	1.517±0.861
Lymph nodes	0.32±0.55	0.076±0.022	0.062±0.015	0.058±0.016
Whole body	73.14±7.56	55.16±1.87	47.39±1.52	38.41±3.02

%IA: % of injected activity



Supplemental Figure 1. Blood clearance of [^{68}Ga]Ga-NOTA-anti-MMR-sdAb in wild type mice.



Supplemental Figure 2. Biological half-life of [^{68}Ga] NOTA-anti-MMR-sdAb was calculated to be 2.49 h.