Preclinical Development

Regression of Human Prostate Cancer Xenografts in Mice by AMG 212/BAY2010112, a Novel PSMA/CD3-Bispecific BiTE Antibody Cross-Reactive with Non-Human Primate Antigens

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Abstract
For treatment of patients with prostate cancer (PCa), we developed a novel T cell-engaging (BiTE) antibody designated AMG 212 or BAY2010112 that is bispecific for prostate-specific membrane antigen (PSMA) and the CD3 epsilon subunit of the T cell receptor complex. AMG 212/BAY2010112 induced target cell-dependent activation and cytokine release of T cells, and efficiently redirected T cells for lysis of target cells. In addition to Chinese hamster ovary cells stably expressing human or cynomolgus monkey PSMA, T cells redirected by AMG 212/BAY2010112 also lysed human PCa cell lines VCaP, 22Rv1, MDA PCa 2b, C4-2, PC-3-huPSMA, and LnCaP at half maximal BiTE concentrations between 0.1 and 4 ng/mL (1.8–72 pmol/L). No lysis of PSMA-negative human PCa cell lines PC-3 and DU145 was observed. The subcutaneous (s.c.) formation of tumors from PC-3-huPSMA cells in NOD/SCID mice was significantly prevented by once daily intravenous (i.v.) injection of AMG 212/BAY2010112 at a dose level as low as 0.005 mg/kg/d. Rapid tumor shrinkage with complete remissions were observed in NOD/SCID mice bearing established s.c. 22Rv1 xenografts after repeated daily treatment with AMG 212/BAY2010112 by either the i.v. or s.c. route. Of note, 22Rv1 tumors were grown in the absence of human T cells followed by intraperitoneal injection of T cells 3 days before BiTE treatment. No effects on tumor growth were observed in the absence of human T cells or AMG 212/BAY2010112. On the basis of these preclinical results, AMG 212/BAY2010112 appears as a promising new BiTE antibody for the treatment of patients with PSMA-expressing PCa.

Introduction
Prostate cancer (PCa) is the most common type of cancer in men in the United States with estimated 192,000 new cases and 27,400 deaths in 2009 (1), and is the second leading cause of cancer death in men after lung cancer. Relapse after primary therapy by radical prostatectomy remains a common problem in PCa patients because an estimated 30% to 40% of men experience recurrence within 10 years (2, 3). There is a critical need for new targeted agents in this PCa patient population and for those patients who become hormone refractory and require chemotherapy.

Antibodies that target PSMA are a potential addition to the armamentarium of PCa therapies. Developments comprise naked humanized or human IgG1 antibodies, and antibodies of enhanced activity such as antibody drug conjugates and radioimmunoconjugates (4). PSMA is also an established marker for imaging of residual PCa and metastases using the radiolabeled diagnostic antibody ProstaScint (5). The antigen is expressed in a high percentage of PCa patients across all stages of the disease (6). A highly promising approach to enhance the anticancer activity of antibodies is the engagement of T cells by using bispecific antibodies and related constructs. One example is blinatumomab, a CD19/CD3-bispecific antibody construct of the BiTE (for bispecific T cell engager) class that has shown high response rates and an acceptable benefit/risk profile in patients with B-cell lineage lymphoma (7) and leukemia (8). The group of Elsasser–Beile was first to design and characterize PSMA/CD3-bispecific antibodies of the so-called diabody format (9–11). While such bacterially expressed diabodies showed conditional T cell activation, redirected PCa cell lysis and activity against outgrowing xenografts in mouse models, their murine nature and highly restricted species cross-reactivity of the CD3 binding moiety greatly limit nonclinical safety assessment.

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Here, we characterized AMG 212/BAY 2010112, the first PSMA/CD3-bispecific single-chain antibody construct that (i) is very close in its amino acid sequence to human germline Ig-V segments, (ii) is cross-reactive to human and non-human primate PSMA and CD3 antigens, (iii) is highly active against PCA tumor formation as well as established PCA xenograft tumors in a novel mouse model, and (iv) is meeting essential requirements as are desired for clinical development of novel antibody-based therapeutics.

Materials and Methods
Expression, production, and purification of AMG 212/BAY2010112
AMG 212/BAY2010112 is a single-chain bispecific antibody construct comprising of an anti-PSMA and an anti CD3 scFv, both reacting with human and cynomolgus targets. AMG 212/BAY2010112 was constructed by recombinant DNA technology and produced in supernatants from stably transfected Chinese hamster ovary (CHO) cells. Purification of the monomeric protein was done in a 2-step process using Ni chelate chromatography followed by gel filtration as described for an EGF receptor-specific BiTE antibody in ref. 12.

Affinity determination on target positive cell lines using Scatchard evaluation
For affinity analysis, saturation-binding experiments were conducted using a monovalent detection system (anti-His Fab/Alexa 488, Micromet GmbH) to determine monovalent binding of AMG 212/BAY2010112 to PSMA or CD3 positive cell lines. The human PCA cell line LNCaP, CHO cells transfected with human PSMA (CHO-huPSMA), CHO cells transfected with cynomolgus monkey PSMA (CHO-cyPSMA), the human T cell line HPB ALL and the rhesus monkey T cell line 4919 LnPx (kindly provided by Prof. Fickenscher, University Erlangen-Nuernberg, Germany; ref. 13) were used. Two to 5×10⁴ cells of the respective cell line were incubated with 50 μL from a 1:3 dilution series (starting at 800 nmol/L) of AMG 212/BAY2010112, followed by 45 minutes of incubation on ice, and 1 residual washing step. Subsequently, cells were incubated for 30 minutes with 30 μL of an anti-His Fab/Alexa488 solution (30 μg/mL). After 1 washing step, cells were resuspended in 120 μL fluorescence-activated cell sorting (FACS) buffer and analyzed using FACS flow or a FACS Canto machine (Becton Dickinson), and Cell quest software (GraphPad Software). Data are means from 3 experiments from at least 2 different sets of experiments.

Epitope determination using linear peptide mapping
The PSMA-binding scFv of AMG 212/BAY2010112 was subjected to an epitope mapping approach based on peptide scanning (Pepscan). 693 different 15-mer peptides were synthesized that span the entire extracellular amino acid sequence of human PSMA and overlap with each neighboring 15-mer peptide by 14 amino acids. These peptides were synthesized directly in 455-well peptide arrays. For this experiment, the anti-PSMA scFv of AMG 212/BAY2010112 was produced in Escherichia coli and crude periplasmic extracts were used for the analysis. The scFv was incubated on polypropylene cards containing the covalently linked peptides and specific binding was detected and calculated as firstly described in ref. 14.

Cell lines and cell culture
LNCaP, 22Rv1, PC-3, and CHO-dhfr- (CHO) were purchased from DSMZ (Germany), VcaP, and MDA PCa 2b from ATCC, and C4-2 from ViroMed Laboratories (Minnetonka, Minnesota), respectively. The cell lines were obtained before the experiment series and analyzed for authenticity by the respective cell bank (DNA-fingerprinting techniques such as short tandem repeat profiling). No additional identification was conducted in-house. The 22Rv1 and C4-2 were cultured in RPMI 1640 and 10% fetal calf serum (FCS; Invitrogen), LNCaP in Iscove’s Basal Medium (Biochrom AG) and 10% FCS, VcaP in Dulbecco’s Modified Eagle’s Media (Biochrom AG), and 10% FCS. MDA PCa 2b cells were cultured in F-12 K- Nutrient Mixture (Invitrogen) and 20% FCS supplemented with 5 mL Insulin-Transferrin-Selenium-Solution (Invitrogen), 10 ng/mL recombinant human EGF (MBL), and 100 pg/mL hydrocortisone (Sigma-Aldrich). Cell lines were cultured at 37° C in a 5% CO₂ chamber.

PC-3 were stably transfected with the human PSMA cDNA (PC-3-huPSMA) using the eukaryotic expression vector pEF-puromycin (15), CHO cells stably expressing human (CHO-huPSMA) or cynomolgus monkey PSMA (CHO-cyPSMA) were generated by transfecting cells with plasmids containing the respective cDNAs. Selection of clones and amplification of expression was conducted in the presence puromycin (0.8 μg/mL) and methotrexate (20 nmol/L).

PK analysis in mice
Female BALB/c mice received a single intravenous (i.v.) bolus injection of 0.1, 0.3, or 1 mg/kg AMG 212/BAY2010112 or were subcutaneous (s.c.) injected with a single dose of 0.3 mg/kg AMG 212/BAY2010112. Whole blood samples were collected at the following times: 0 (predose), 0.083, 0.167, 0.5, 1, 2, 4, 7, 24, and 48 hours after administration. AMG 212/BAY2010112 serum concentrations were determined by an electrochemiluminescence-based assay. A polyclonal goat anti-AMG 212/BAY2010112 serum was coated to a high binding plate (MSD). After blocking overnight with Dulbecco’s Phosphate Buffered Saline containing 5% bovine serum albumin, plates were washed with an ELISA washer. Standards, quality-control samples, and study samples were added to the carbon plate and were subsequently incubated on a rotation shaker for 1 hour at room temperature. After another wash step with the ELISA washer, the detection anti-penta His-biotin-labeled detection antibody (GenScript) was added and incubated for 1 hour.
on a rotation shaker at room temperature. Streptavidin SulfoTag (MSD) was added and the plates were incubated for 1 hour at room temperature, followed by another wash step. Finally, reading buffer T (MSD) was added and the plates were analyzed at the Sector Imager 2400 according to the manufacturer’s instructions. All samples were determined in triplicates. The pharmacokinetic parameters were calculated from the geometric mean plasma concentrations by noncompartmental analysis (16, 17) using the KinEx program version 3.0 (Bayer Pharma AG, 2010).

Cytotoxicity, T cell activation, and cytokine release

Redirected T cell cytotoxicity was assayed by flow cytometry using human and cynomolgus monkey peripheral blood mononuclear cells (PBMC) as effecter cells and PSMA-positive human PCA cell lines or PSMA-transfected CHO cells as targets. PBMC were isolated by Ficoll density gradient centrifugation and if applicable enrichment for CD3+ T cells was conducted by using the Pan T Cell Isolation Kit (Miltenyi Biotech) according to the manufacturer’s instructions. Effector cells were cocultivated with target cells labeled with Vybrant DiO (Invitrogen) at effector-to-target (E:T) cell ratios of 5:1 or 10:1 with increasing concentrations of AMG 212/BAY2010112 for 24 hours and cell lysis was monitored by nuclear uptake of propidium iodide by flow cytometry (12).

Activation of CD4+ and CD8+ T cell subsets was determined by using phycoerythrin (PE)-labeled anti-CD4 and allophycocyanine (APC)-Cy7-labeled anti-CD8 antibodies, PE-Cy7-conjugated anti-CD69 and APC-conjugated anti-CD25 were used to characterize activated T cells. All fluorescently labeled antibodies were purchased from BD Biosciences (Heidelberg). For staining of cell surface proteins, cells were incubated with the appropriate antibody mixtures for 30 minutes at 4°C and samples were analyzed by flow cytometry.

Cytokine concentrations were determined in supernatants of cytotoxicity assays by use of cytokometric bead arrays (CBA-kit, BD Biosciences) in accordance to the manufacturer’s protocol.

Animal tumor studies

In vivo studies to evaluate antitumor activity were conducted in, NOD.CB17-Prkdcsidcd/J mice (Charles River). Mice were housed in individually ventilated cages with laboratory chow and water available ad libitum. All experiments were conducted according to the German Animal Protection Law with permission from the responsible local authorities.

In the tumor formation study, 5 x 10⁶ PC-3-huPSMA cells were injected s.c. with or without PBMC obtained from a single healthy human donor at an E:T cell ratio of 1:2 into the flank of female nude or severe combined immunodeficient (NOD/SCID) mice (n = 8). Mice were treated once daily from the day of tumor/ PBMC injection with 5, 0.5, 0.05 and 0.005 mg/kg AMG 212/BAY2010112 BiTE antibody for 10 days by i.v. bolus injection into the lateral tail vein. Controls included 2 vehicle-treated groups (tumor cells injected with or without human PBMC). Treatment efficacy in terms of tumor formation and growth was determined by external caliper measurements, and tumor volumes were calculated using a standard hemi-ellipsoid formula: \( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \).

For the established tumor model, male NOD/SCID mice were irradiated with a single dose (2 Gy) of 137Cs γ-ray before s.c. injection of 22Rv1 (5 x 10⁶) tumor cells dispersed in medium with 50% Matrigel (Cultrex, R&D Systems) before injection. Mice were irradiated to facilitate engraftment of the injected human T cells. Animals (n = 8) were allocated to treatment groups when tumors had reached a volume of approximately 200 mm³ and were treated with a single i.v. bolus injection of anti-asialo GM1 rabbit antibody (WAKO) into the lateral tail vein 1 day before human T cell injection to deplete murine NK cells. In vitro activated and expanded human T cells isolated from the blood of a single donor (T cell activation/expansion kit, Miltenyi Biotech) were injected into the peritoneal cavity on day 11 after tumor cell injection. One control group of animals (n = 5) did not receive human T cells. Animals received AMG 212/BAY2010112 or vehicle once daily by either s.c., or i.v. bolus injection into the lateral tail vein for a total of 28 days starting at day 14 posttumor cell inoculation. Growth of tumors was determined as described above.

Statistical analysis

Data were analyzed by ANOVA, and differences in tumor volumes of the AMG 212/BAY2010112 BiTE-treated groups to the vehicle control group injected with human effecter cells were assessed with a Dunnett’s post-test. P < 0.05 was considered to be statistically significant. All statistical analyses were conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software).

Results

Generation of human PSMA/CD3-bispecific BiTE antibody AMG 212/BAY2010112

The PSMA/CD3-bispecific BiTE antibody AMG 212/BAY2010112 as shown in Fig. 1A was purified in larger amounts to apparent homogeneity (Fig. 1B). It showed one major protein species (95.6%) of defined charge upon analytical high-resolution cation exchange chromatography (Fig. 1C). Only the monomeric form of AMG 212/BAY2010112 was used for further in vivo and in vitro characterization. The BiTE antibody showed a high stability in human plasma at 37°C where after incubation for 24 hours EC₅₀ values for redirected target cell lysis were not significantly different to untreated controls.

Bispecific binding of AMG 212/BAY2010112 to CD3 and a defined epitope of PSMA

Saturation binding curves of AMG 212/BAY2010112 to CHO cells expressing either human or cynomolgus monkey PSMA and to T cells of human or cynomolgus monkey
origin were used to determine equilibrium binding constants ($K_D$) of AMG 212/BAY2010112. As shown in Fig. 2A, AMG 212/BAY2010112 bound to CD3 and PSMA of both species. $K_D$ values for human and cynomolgus monkey CD3 were 9.4 ± 4.3 nmol/L and 16.3 ± 2.1 nmol/L, respectively, and 47.0 ± 8.1 nmol/L and

Figure 1. Characteristics of purified AMG 212/BAY 2010112. The domain arrangement of AMG 212/BAY2010112 is schematically shown in A. Purified AMG 212/BAY2010112 was separated by SDS-PAGE and stained with Coomassie Blue (B) and subjected to high-resolution cation exchange chromatography (C) to show purity and charge homogeneity. The elution profile of purified AMG 212/BAY2010112 from the cation exchange column was recorded by absorption at 280 nm. The calculated molecular weight of AMG 212/BAY2010112 is 55 kDa.

Figure 2. Binding of AMG 212/BAY2010112 to human and cynomolgus monkey PSMA and CD3 antigens. Increasing concentrations of monomeric AMG 212/BAY2010112 were incubated with 10,000 cells of the indicated cell lines. Cell-bound BITE antibody was detected by flow cytometry using a monovalent antihexahistidine Fab antibody fragment covalently coupled to ALEXA 488. Binding signals are given as median relative fluorescence intensity (RFI). A, hyperbolic binding curves were plotted and half-maximal binding concentrations ($K_D$) were calculated using the algorithm by George Scatchard. Exemplary binding curves are depicted. $K_D$ values with standard deviations were determined from 3 independent experiments per cell line. B, the epitope on human PSMA recognized by AMG 212/BAY2010112 was determined by binding of the anti-PSMA single-chain antibody moiety of AMG 212/BAY2010112 to a panel of 15-mer peptides permuted by 1 amino acid that spanned the entire extracellular domain of human PSMA. Binding signals for peptides in the ELISA assay are given as relative signal units. C, the position of the most reactive peptide is highlighted in the crystal structure of human PSMA (20).
212.6 ± 29 nmol/L for human and cynomolgus monkey PSMA, respectively.

To map the binding site of AMG 212/BAY2010112 to human PSMA, overlapping peptides of the extracellular domain of PSMA were synthesized and tested for binding of AMG 212/BAY2010112 using a PepBlot (18, 19). The highest binding signal of AMG 212/BAY2010112 was seen with a peptide corresponding to amino acid residues 325 to 348 of human PSMA (Fig. 2B). According to the published crystal structure of PSMA (20), this peptide localizes to a small loop structure on the apical side of the protein facing away from the plasma membrane (Fig. 2C). The sequence of this peptide is identical between human and cynomolgus monkey PSMA, which explains the cross-species binding of AMG 212/BAY2010112 to PSMA.

**Human/cynomolgus monkey cross-reactivity of AMG 212/BAY2010112 for T cell activation, cytokine release, and redirected lysis**

The pharmacologic effects of AMG 212/BAY2010112 on effector T cells and PSMA-expressing target cells were analyzed in vitro using cell coculture assays. AMG 212/BAY2010112 activity was compared in both human and cynomolgus monkey cell-based assay systems to investigate whether the cynomolgus monkey can serve as relevant species for pharmacologic and toxicological safety assessments.

The dose-dependent activation of CD4+ and CD8+ T cell populations by AMG 212/BAY2010112 was analyzed using FACS by the appearance of the immediate-early and late activation markers CD69 and CD25, respectively, after 48 hours of incubation in the presence of human LNCaP PCa cells (Fig. 3A). At the maximum, between 60% to 80% of CD4+ and CD8+ T cells of both species were activated, whereas less than 10% of T cells were found activated when freshly isolated. Values for half-maximal effective concentration (EC50) of T cell activation ranged between 3.4 and 6.7 ng/mL of AMG 212/BAY2010112 for human and between 13.7 and 21.2 ng/mL for cynomolgus monkey cell cocultures. The largest difference between human and cynomolgus monkey T cells was observed with the CD4+/CD25+ T cell population where EC50 values differed by 6.2-fold. No T cell activation was observed in the absence of PSMA-expressing target cells (data not shown).

Next, the AMG 212/BAY2010112 dose-dependent release of 6 different cytokines by activated T cells in the presence of target cells was compared. The cytokine release profiles were very similar in human and cynomolgus monkey cell coculture systems with absolute amounts of cytokines reaching approximately 2-fold higher levels in the human system. Highest concentrations were observed for interferon-γ, which reached 3 to 6 ng/mL in cell culture medium after 48 hours (Fig. 3B). Intermediate increases reaching levels of 1 to 1.5 ng/mL were observed for TNF-α, IL-2 and IL-10. For technical reasons, IL-10 levels could not be determined in cynomolgus monkey cocultures, whereas IL-5 levels were not detected in human cocultures. Little or no increase was observed for IL-4 and IL-6. As observed previously, cytokine release of T cells was observed only in the presence of target cells (data not shown), which is one hallmark of BiTE antibodies (21).

The dose-dependent redirected lysis of target cells by AMG 212/BAY2010112 was investigated with CHO cells, which expressed either human or cynomolgus monkey PSMA, to have the same cellular background of target cells. Overall, potent lysis of transfected CHO cells was observed with EC50 values ranging across species between 0.37 and 6.69 ng/mL (Fig. 3C). In homologous assays where T cells and PSMA from the same species were used, an 18-fold higher concentration of AMG 212/BAY2010112 was required to reach half maximal lysis in the cynomolgus monkey system compared with the human system. Notably, target cells expressing human PSMA were more potently lysed by either human or cynomolgus monkey T cells than those expressing cynomolgus monkey PSMA, which may be attributed to the somewhat lower affinity of AMG 212/BAY2010112 to cynomolgus monkey PSMA (Fig. 2A). Human T cells showed a 5-fold higher potency of redirected lysis with AMG 212/BAY2010112 than cynomolgus monkey T cells, regardless whether human tumor cells or CHO cells expressing either human or cynomolgus monkey PSMA were used. Taken together, AMG 212/BAY2010112 showed dose-dependent activity for T cell activation, cytokine release and redirected lysis with human and cynomolgus monkey T cells, and reacted with PSMA antigen from both species.

**Redirected lysis of human PCa lines by AMG 212/BAY2010112**

To assess the potency of redirected lysis by AMG 212/BAY2010112 with target cells naturally expressing PSMA, the 7 human PCa cell lines VCaP, 22Rv1, MDA PCa 2b, C4-2, PC-3, DU145, and LNCaP were procured. FACS staining showed that, except for PC-3 and DU145 cells, all PCa cell lines expressed detectable levels of PSMA. Surface densities of PSMA were quantified and ranged from 1.2 × 10⁶ (VCaP) to 5 × 10⁷ (PC-3-huPSMA) PSMA molecules per cell (see Fig. 4B). Target cells were incubated in the presence of increasing concentrations of AMG 212/BAY2010112 with a 10-fold excess of unstimulated PBMC as effector cells and lysis was measured after 48 hours by nuclear uptake of propidium iodine via FACS. All 5 PCa cell lines, which naturally express PSMA as well as PC-3-huPSMA cells (stably expressing a human PSMA cDNA), were lysed with EC50 values ranging between 0.1 and 4 ng/mL (i.e., 1.8–64 pmol/L) AMG 212/BAY2010112 (Fig. 4A). No lysis of parental PSMA-negative PC-3 cells was observed. EC50 values for redirected lysis correlated with the number of surface expressed PSMA molecules per cell in a statistically significant manner (Fig. 4B).

**Pharmacokinetics of AMG 212/BAY2010112 in BALB/c mice**

Pharmacokinetic parameters of AMG 212/BAY2010112 were determined after i.v. bolus administration of 0.1,
0.3, and 1 mg/kg to BALB/c mice. A dose proportional increase of serum concentrations was observed. Serum clearance was low and amounted to approximately 0.3 L/(kg/h) independently of the dose. An intermediate volume of distribution at steady state of approximately 1 L/kg was observed. This indicates that the BiTE antibody did not only distribute into the extravascular space (as observed for IgGs) but also into tissues, which resulted in a considerably extended terminal half-life of approximately 8 hours.

After s.c. administration of 0.3 mg/kg to mice, maximum serum concentrations of approximately 64 μg/L were reached 2 hours after administration. Bioavailability was 18%, indicating significant metabolism during absorption via the lymphatic system. Pharmacokinetic parameters after i.v. and s.c. bolus administration to mice are summarized in Table 1.

**Inhibition of tumor growth and tumor regression by AMG 212/BAY2010112-directed human T cells in immunodeficient mice**

Following the characterization of AMG 212/BAY2010112 in vitro, its efficacy on the inhibition of tumor growth was investigated in mouse xenograft models. AMG 212/BAY2010112 was first studied for its preventive effect in a tumor outgrowth model. PC-3-huPSMA cells were mixed with unstimulated human PBMC shortly before injection and mixtures were administered s.c. to immunodeficient NOD/SCID mice. Immediately following tumor cell inoculation, mice were treated i.v. with AMG 212/BAY2010112...
at doses ranging from 5 mg/kg down to 0.005 mg/kg by once daily injection for 10 consecutive days. Efficacy was evaluated by monitoring tumor formation. A significant and dose-dependent delay of tumor formation/growth was observed with tumor growth inhibition after a monitoring period of 43 days ranging from 86% (0.005 mg/kg/d) to 99% (5 mg/kg/d) compared with the vehicle plus PBMC control (Fig. 5A). The inhibition was statistically significant even at the lowest dose of 0.005 mg/kg, which corresponded to only 0.1 ng AMG 212/BAY2010112 per mouse/d. AMG 212/BAY2010112 completely prevented tumor formation in 1 and 2 of 8 animals at doses of 0.5 or 5 mg/kg/d, respectively, whereas small nodules became visible in some animals on Days 46 and 53, respectively.

We next addressed the question whether AMG 212/BAY2010112 could affect established PCa xenografts. A new study design was established in which human effector T cells were delivered shortly before initiation of BITE treatment by intraperitoneal (i.p.) injection into tumor-bearing NOD/SCID mice. Previous experiments have shown that i.p.-delivered human T cells enter the circulation of mice and remain detectable for approximately 2 weeks (22). For the present study, human 22Rv1 PCa xenografts were grown s.c. in male NOD/SCID in the absence of human T cells until they had reached a volume of 180 to 200 mm³. Then, human T cells expanded in vitro for 18 days were injected i.p. and treatment with AMG 212/BAY2010112 was initiated 3 days after the adoptive T cell transfer by once daily i.v. or s.c. injections with doses of 0.5 or 2.5 mg/kg, respectively. To achieve similar drug exposures for i.v. and s.c. administrations a 5 times higher dose was given s.c. as based on the 18% bioavailability of AMG 212/BAY2010112 after s.c administration (see Table 1). Complete tumor remissions were observed in 3/8 animals of the s.c.-treated group after necropsy, whereas small nodules with sizes less than 50 mm³ were detected in the residual animals. In the i.v.-treated group small nodules of less than 50 mm³, most likely representing fibrous tissue remnants, were detected in 3/8 animals, whereas small tumors remained in the other animals. Unrestricted tumor growth occurred in animals treated with human T cells alone or a vehicle. In summary AMG 212/BAY2010112 very potently inhibited growth of PSMA expressing human PCa xenografts in mice, which eventually led to complete remission in some animals after s.c. application.

Discussion

In the present study, the anti-PSMA/anti-CD3 single-chain antibody construct AMG 212/BAY2010112 was examined for its in vitro and in vivo pharmacologic

Table 1. Pharmacokinetic parameters of AMG 212/BAY2010112 after i.v. and s.c. administration to BALB/C mice

<table>
<thead>
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<th>Administration</th>
<th>i.v. bolus</th>
<th>s.c. bolus</th>
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<td>Dose (mg/kg)</td>
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<td>9.7</td>
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<tr>
<td>F (bioavailability) (%)</td>
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<td>100</td>
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NOTE: Serum levels of AMG 212/BAY2010112 after single bolus i.v. administration of 0.1, 0.3, and 1 mg/kg and after single bolus s.c. administration of 0.3 mg/kg to female BALB/c mice were determined as described in Materials and Methods. Abbreviations: AUC, area under the curve; AUCnorm, normalized AUC; CLmatrix, serum clearance; Cmax norm, maximum serum levels; Vss, volume of distribution at steady state; t1/2, terminal serum half-life (beta); n.a., not applicable.
properties and its suitability for nonclinical safety studies
with non-human primates.

Cell binding studies revealed that CD3 antigens of human and
cynomolgus monkey origin were bound by AMG 212/BAY2010112 with similar equilibrium dissociation constants in the nanomole per liter range. The differences seen can be compensated in respective toxicology studies by studying higher dose levels of the BiTE antibody in cynomolgus monkeys. Importantly, qualitative responses of human and cynomolgus monkey T cells to AMG 212/BAY2010112 were largely comparable with respect to the magnitude of target cell lysis, T cell activation, and cytokine release, supporting the use of cynomolgus monkeys as a relevant species for the nonclinical safety assessment of AMG 212/BAY2010112. Respective nonclinical safety studies are currently ongoing. The first in human study (phase I) is planned in patients with advanced castration-resistant PCa but expect AMG 212/BAY2010112 to be beneficial also for patients in earlier stages of the disease.

Previous work has provided in vitro proof of concept that PSMA is a useful target for lysis of PSMA-expressing PCa cells by redirected T cells using bispecific diabodies (9–11). These are produced in E. coli, are of murine sequence and their CD3-binding moiety, which is related to the anti-CD3 antibody OKT-3, does not react with T cells from cynomolgus monkeys (23). Moreover, information of such diabodies concerning pharmaceutical properties such as productivity, serum stability, aggregation behavior, and charge homogeneity is scarce. Using the clinically validated BiTE format, we generated a molecule, which appears for a number of reasons better suited for development as a human therapeutic than currently existing diabodies: (i) we used anti-PSMA and anti-CD3 single chain antibodies that are very close in their amino acid sequence to human germline Ig-V segments, an established feature reducing potential immunogenicity of antibodies. (ii) We used single-chain antibodies that bind to epitopes shared by human and monkey target antigens providing the basis for nonclinical safety assessment of AMG 212/BAY2010112 in a pharmacologically relevant species. (iii) AMG 212/BAY2010112 was selected from a panel of candidate molecules as lead candidate for its superior activity and CMC related properties. Activity-related selection parameters comprised the cytotoxic activity with activated human T cells and unstimulated human PBMC as effector cells directed against human PSMA transfected CHO cells and human PCa cell lines expressing native PSMA. Cytotoxic activity in the macaque system was assessed with macaque PSMA transfected CHO cells and a macaque T cell line. CMC-related selection parameters comprised production yield, protein
homogeneity in high-resolution ion exchange chromatography, stability in human plasma, thermal stability, and dimer conversion. Compared with published PSMA/CD3-bispecific diabodies, which have EC50 values for redirected lysis of approximately 0.25 nmol/L (15 ng/mL; refs. 9, 10), AMG 212/BAY2010112 is considerably more potent with EC50 values ranging from 1.8 to 72 pmol/L (0.1 and 4 ng/mL) depending on the cell line. Only recently, a newly constructed diabody was published with an EC50 value of approximately 23 pmol/L (1.4 ng/mL) using C4-2 target cells (11). (iv) Finally, we observed that AMG 212/BAY2010112 can mediate remission of 22Rv1 PCa xenografts, whereas published PSMA/CD3-bispecific diabodies were thus far only shown to inhibit tumor formation. On the basis of all of the above features, we believe that AMG 212/BAY2010112 meets all properties required for development of a potent novel human therapeutic.

This is also the first report on a novel in vivo study design for analyzing the activity of T cell-engaging antibodies against established tumors in mice. Most importantly, tumors were grown s.c. to an average size of 200 mm3 in NOD/SCID mice in the absence of human T cells. Ex vivo expanded human T cells were then injected into the peritoneal space of mice from where they had to enter the peripheral blood compartment to reach the s.c. implanted tumor. Three days following adoptive T cell transfer, dosing of AMG 212/BAY2010112 was started, which triggered a rapid onset of tumor regression. This is consistent with an immediate engagement of peripheral T cells for redirected tumor lysis. The fast onset is different from vaccines and anti-CTLA-4 antibodies, which require days to weeks for mounting a tumor-specific T cell response in man (22). Rapid tumor regression also suggests that both AMG 212/BAY2010112 and i.p.-delivered T cells readily gained access to tumor tissue. In our present study, the s.c. administration of the BiTE antibody appeared to be even more efficacious than the i.v. route. The bioavailability of AMG 212/BAY2010112 in mice after administration of a single s.c. dose was 18%. Preliminary studies indicate that AMG 212/BAY2010112 is bioavailable and pharmacologically active after s.c. application in cynomolgus monkeys. Additional studies are in progress to better understand the activity of AMG 212/BAY2010112 after s.c. administration and evaluate its applicability for treatment of PCa patients.

Disclosure of Potential Conflicts of Interest
M. Friedrich, T. Raum, R. Lutterbuese, M. Voelkel, P. Deegen, D. Rau, R. Kischel, P. Hoffmann, C. Brandl, P. Mueller, M. Fuerget, P.A. Baeuerle, B. Rattel, and P. Kufer are Amgen employees and have received stock. T. Raum, R. Lutterbuese, R. Kischel, P. Hoffmann, P. Kufer have ownership interest (including patents). J. Schuhmacher, R. Finnern, and D. Zopf are Bayer Pharma employees. J.W. Slootstra is employed by Pepscan Presto.

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