Pharmacologic Profile of the Adnectin BMS-962476, a Small Protein Biologic Alternative to PCSK9 Antibodies for Low-Density Lipoprotein Lowering

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ABSTRACT

Proprotein convertase subtilisin/kexin-9 (PCSK9) is an important pharmacological target for decreasing low-density lipoprotein (LDL) in cardiovascular disease, although seemingly inaccessible to small molecule approaches. Compared with therapeutic IgG antibodies currently in development, targeting circulating PCSK9 with smaller molecular scaffolds could offer different profiles and reduced dose burdens. This inspired genesis of PCSK9-binding Adnectins, a protein family derived from human fibronectin-10th-type III–domain and engineered for high-affinity target binding. BMS-962476, an ∼11-kDa polypeptide conjugated to polyethylene glycol to enhance pharmacokinetics, binds with subnanomolar affinity to human. The X-ray co-crystal structure of PCSK9 with a progenitor Adnectin shows ∼910 Å² of PCSK9 surface covered next to the LDL receptor binding site, largely by residues of a single loop of the Adnectin. In hypercholesterolemic, overexpressing human PCSK9 transgenic mice, BMS-962476 rapidly lowered cholesterol and free PCSK9 levels. In genomic transgenic mice, BMS-962476 potently reduced free human PCSK9 (ED₅₀ ∼0.01 mg/kg) followed by ∼2-fold increases in total PCSK9 before return to baseline. Treatment of cynomolgus monkeys with BMS-962476 rapidly suppressed free PCSK9 >99% and LDL-cholesterol ∼55% with subsequent 6-fold increase in total PCSK9, suggesting reduced clearance of circulating complex. Liver sterol response genes were consequently downregulated, following which LDL and total PCSK9 returned to baseline. These studies highlight the rapid dynamics of PCSK9 control over LDL and liver cholesterol metabolism and characterize BMS-962476 as a potent and efficacious PCSK9 inhibitor.

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a 74-kDa protein secreted by liver that regulates hepatic low-density lipoprotein (LDL) receptor (LDLR) activity and controls plasma LDL cholesterol (LDL-C) levels. By virtue of its genetics and biologic mechanism (Abifadel et al., 2003; Cohen et al., 2006), PCSK9 has emerged as an important therapeutic target for discovery and development of new drugs for cardiovascular diseases (Steinberg and Witzum, 2009). PCSK9 binds to the LDLR within its well conserved epidermal growth factor precursor homology domain-A (EGFA) at the cell surface (Kwon et al., 2008). Upon binding, PCSK9 cointernalizes into endosomes and redirects receptor sorting into the lysosomal degradative pathway rather than recycling to the surface (Zhang et al., 2007). If PCSK9 binding is inhibited, the biologically important cargo delivery function of the receptor is increased, the liver intracellular cholesterol pool is augmented, and plasma LDL-C is decreased.

Because small molecule approaches targeting PCSK9 have proven difficult to date, unlike for some members of the proprotein convertase gene family (Seidah et al., 2008), drug discovery attention has focused upon antisense oligonucleotides (Graham et al., 2008; Fitzgerald et al., 2013) or monoclonal antibody (mAb) based biologics (Chan et al., 2009; Ni et al., 2011; Liang et al., 2012; reviewed by Seidah 2009).
and Prat, 2012). Structure-function analysis suggests targeting the EGFA-interacting region of PCSK9 for pharmacological inhibition (Zhang et al., 2007). The EGFA site involves residues 367–381 covering about 500 Å² of PCSK9 surface (Kwon et al., 2008) and includes the D374Y locus, a gain-of-function mutation that increases activity 10-fold (Lagace et al., 2006). These high-affinity mAbs were found to cover a somewhat larger region at or near the EGFA binding site (Chan et al., 2009; Liang et al., 2012). These and other PCSK9 mAbs (Ni et al., 2011; Stein et al., 2012) lowered LDL in animal studies and human clinical trials. At the other end of the size spectrum, Zhang et al. (2014) recently described a linear peptide of 13 amino acids that contacted about 400 Å² of PCSK9 adjacent to the EGFA interaction site and provided full-length LDLR binding, and potent biologic effects in cells, binding affinity to human PCSK9, displacement of EGFA and PK enhancement.

Materials and Methods

PCSK9 Adnectin Selection, Expression, and Purification. Generation of the Adnectins followed procedures described previously (Parker et al., 2005; Lipovsek, 2011). Briefly, an Adnectin DNA library of high diversity (2.3 × 10^12) produced by randomizing the 3 variable loops (denoted BC, DE, and FG) of 10Fn3 was taken through multiple rounds of mRNA display. In each round, the library was transcribed in vitro, translated using rabbit reticulocyte, and each encoding mRNA was fused to its own Adnectin via a puromycin linkage. Reverse transcription was used to generate a cDNA tail on each Adnectin, and during selection, Adnectins that bound human PCSK9 were amplified by polymerase chain reaction to serve as template for the next round. Cycles of selection and amplification were continued, sequenced, and assayed. Lead candidates were chosen based on biophysical properties and inhibition potency in PCSK9: EGFA binding and cell-based assays. The progenitor Adnectin, clone 1459D05, had the following amino acid sequence: GVSDVPRDLEVVAATPTSSLISWPPFSSHHGYGYTIRTGETGNGNSPVQFETVPQPKG TAT ISLKGPGVDYTVVAYEVPPKHSGYHRPSINYRTEIDKP SQHDHHHHH.

To further enhance binding affinity, the 1459D05 sequence was affinity matured using an optimization library with repeated selections of increasing stringency to favor clones with tighter affinity and slower off-rate. The PCSK9 Adnectin BMS-962476 is the optimized variant of 1459D05 with higher binding affinity and potency in functional assays. BMS-962476 has a protein molecular mass of 11.3 kDa, comprising 103 residues with the following sequence: GVSDVPRDLEVVAATPTSSLISWPPFSSHHGYGYTIRTGETGNGNSPVQFETVPQPKG TAT ISLKGPGVDYTVVAYEVPPKHSGYHRPSINYRTEIDKP SQHDHHHHH.

BMS-962476 also contains a 40-kDa, 2-branched polyethylene glycol moiety conjugated via standard maleimide chemistry at the penultimate C-terminal cysteine residue (PEGylated Adnectin). The C-terminal His6 purification-tagged form of the PEGylated protein was also studied (referred to as BMS-962476 his6). The non-PK enhanced, non-PEGylated form of this Adnectin (referred to as BMS-962476 his6, no PEG) exhibits very high thermal stability (T_max = 81°C) and solubility with monomeric behavior by gel filtration chromatography.

PCSK9 Protein Expression and Purification. Recombinant full-length human PCSK9 (31–692) and truncated human PCSK9 (53–451, for crystallography) and full-length PCSK9 from cynomolgus monkey (cyto), Guinea pig, and mouse cDNA sequences were produced and purified from baculovirus expression or stably transfected mammalian cell systems by standard molecular biology and chromatography techniques, essentially as described previously (Benjannet et al., 2010).

Biophysical Assessment of Adnectins. Size exclusion chromatography was used to profile solution monomeric behavior. Size exclusion chromatography runs were performed on an Agilent 1200 system (Agilent Technologies, Santa Clara, CA) using Superdex-75 10/300 column (GE Healthcare, Life Sciences, Piscataway, NJ). Mobile phase for all SEC runs consisted of 100 mM NaPO4, 100 mM NaSO4, 150 mM NaCl, pH 6.8, and data analysis was performed using Chemstation software package provided by Agilent Technologies. Differential scanning calorimetry (DSC) was performed on samples dialyzed into phosphate-buffered saline (PBS) and normalized to 0.5 mg/ml before run. DSC sample runs were conducted at a scan rate of 60°C/hour from 15 to 95°C on a MicroCal VP-DSC (GE Healthcare, Life Sciences). Data analysis was performed using Origin software package (GE Healthcare, Life Sciences).

PCSK9/Adnectin Complex Crystallography and Structure Determination. Purified human PCSK9 (53–451) and Adnectin 1459D05 were mixed in a 1:3 molar ratio and incubated on ice for 5 hours with gentle mixing every 30 minutes. To remove excess
unbound Adnectin, the complex was run over Superdex-75 (16/60) column equilibrated in 25 mM HEPES pH 7.5, 0.2 M NaCl, and 5% glycerol at flow rate 1 ml/min; peak fractions containing 1:1 Adnectin: PCSK9 complex were pooled and concentrated. The sample was clarified by centrifugation and concentrated to 10 mg/ml for use in crystallization experiments. Crystals of PCSK9/1459D05 were obtained by hanging drop vapor diffusion method at 22°C by equilibrating the complex against a reservoir solution containing 22% (v/v) PEG 200, 1% (v/v) ethylene glycol, and 0.1 M MES pH 6.5. Crystals were harvested by flash-cooling in liquid nitrogen using 30% (v/v) PEG200 and 0.1 M MES pH 6.5 as a cryoprotectant.

X-ray data for the cocystal complex were collected at beamline 17ID at IMCA-CAT of the Advanced Photon Source at Argonne National Laboratory (Lemont, IL). The wavelength used was 1.00 Å and the detector was a MAR-165 CCD (Marresearch GmbH, Norderstedt, Germany). The diffraction images were indexed, integrated, and scaled with D*TREK (Pflugrath, 1999). The structure was determined by molecular replacement using the program PHASER (McCoy et al., 2007). Two different PDBs served as search models for molecular replacement: 1) PDB ID 2W2M (Bottomley et al., 2009) for PCSK9 and 2) PDB ID 1FPN (Leahy et al., 1996) as search model for the Adnectin. In the latter, the variable loops and N and C termini were removed before running molecular replacement. Model building was done for one monomer of the PCSK9-Adnectin complex, which was then used as search model to find the second complex in the asymmetric unit. The model was built using the programs O (Shen et al., 2004) and COOT (Emsley and Cowtan, 2004). The structure was initially refined using Refmac5 (CCP4 Suite) and the final cycles using autoBUSTER (Global Phasing LTD, Cambridge, UK). Figures were generated using gpmol (PyMOL Molecular Graphics System, v1.5.0.5; Schrödinger, LLC, New York, NY). The final structure has a resolution of 2.69 Å, an R factor = 19.9% and Rfree = 23.08%. Coordinates and structure amplitudes are deposited as PDB ID 4OV6.

**Measurement of Binding Affinity and Kinetics.** Binding studies were conducted in PBS buffer at pH 7.4 unless otherwise noted. Adnectin binding to immobilized PCSK9 was measured by biolayer interferometry (Octet Red 96, using Superstrepavidin sensor tips; ForteBio, Menlo Park, CA). Association and dissociation events were measured in real time for a series of Adnectin concentrations with biotinylated full-length PCSK9 captured on sensor tips. Binding curves were globally fit to produce values for $K_D$, $k_{on}$, and $k_{off}$. With the use of an alternative solid phase format, Adnectin binding affinity for immobilized PCSK9 was assessed using surface plasmon resonance (SPR) in PBS + 0.05% Tween-20 using a ProteoOn-XPR (Bio-Rad, San Francisco, CA), with data reduction using the Langmuir 1:1 interaction model and constant parameter fitting with ProteoOn Manager software. Affinity was also measured by solution phase methods using the kinetic exclusion assay essentially as described by Drake et al. (2004), which is particularly well suited to measuring subnanomolar affinities. As a final test, affinity was measured using solution-phase isothermal calorimetry (ITC) with a VP-ITC calorimeter (MicroCal/GE Healthcare Life Sciences). ITC, which measures the thermodynamics and stoichiometry of protein:protein binding by quantifying the amount of heat released or absorbed, is in some cases the most accurate method, although systems with subnanomolar affinities at ambient temperature require measurements at higher temperatures with extrapolation to ambient (Doyle, 1997). Although the PEG moeity is positioned away from the presumed target binding region of the Adnectin, in some assay platforms with various proteins the PEG moeity can interfere with binding measurements (Kubetko et al., 2005). Therefore it was relevant to study the non-PEGylated form of the Adnectin and to test multiple different assay platforms.

**LDLR-EGFA Competitive Inhibition Fluorescence Resonance Energy Transfer Assay.** A competitive binding assay based on homogeneous time-resolved fluorescence resonance energy transfer (FRET) was used to measure displacement of the EGFA peptide to PCSK9 protein. EGFA peptide synthesis by solid-phase chemistry and the assay conditions were essentially as described previously (Benjannet et al., 2010). Briefly, the FRET assay buffer was 150 mM NaCl, 5 mM CaCl$_2$, 10 mM HEPES, pH 7.4, and the assay in 384-well plates contained 10 nM PCSK9 (or as specified in data), 0.5 nM europium chelate-labeled anti-PCSK9 mAb-4H5, 400 nM EGFA (biotinyl), 40 nM streptavidin-allophycocyanin, and specified levels of Adnectins. mAb4H5 is a BMS custom antibody produced by Lampire Biologic Laboratories (Pipersville, PA) that recognizes the C-terminal domain of PCSK9. Plates were incubated at 20°C for 16 hours, followed by measurement of time-resolved fluorescence using a PerkinElmer (Waltham, MA) Envision instrument with laser excitation (37/615/665 nm). Data were fitted as nonlinear regression sigmoidal dose response variable slope using logarithm of concentration with GraphPad Prism software (GraphPad Software, La Jolla, CA).

**Cell-Based PCSK9 Uptake and LDLR Functional and Protein Level Assays.** To assay LDLR-dependent PCSK9 uptake into cells, human hepatoma HepG2 cells (HB8065; American Type Culture Collection, Manassas, VA) were incubated for 18 hours in RPMI 1640 media (11835; Invitrogen, Grand Island, NY) containing 5% lipoprotein-deficient serum (LPDS; Intralcip, Frederick, MD; RP054), mevalonate (50 μM), and the statin BMS-423526 (100 nM) to upregulate LDLR expression. Adnectins diluted in media were added at concentrations ranging typically from 0.03 to 300 nM together with fluorescently labeled PCSK9-AF647 at 25 nM. PCSK9 covalently coupled with AF647 fluorophore was prepared using the protocol provided by Molecular Probes (A20173; Invitrogen, Carlsbad, CA). The plates were incubated at 37°C for 4 hours and washed and fixed with 2% formaldehde in the presence of 4 μg/ml of Hoechst DNA stain. The plates were read for intracellular (endosomal) AF647 fluorescence using a Cellomics ArrayScan, and image data were quantified as the mean total intensity of fluorescence per cell from quadruplicate wells with an average read of 600 cells/well. Data were analyzed in GraphPad Prism 4 using nonlinear regression sigmoidal dose response variable slope curvefits for IC$_{50}$ and EC$_{50}$ values.

LDLR functional activity was assayed using HepG2 cells pretreated as described above. After 18 hours, purified PCSK9 (135 nM) was added with Adnectins as indicated in the data (note, higher PCSK9 levels are required to observe functional activity on LDLR by this method). After 2-hour incubation of cells at 37°C, Di-HEL (3'-dioctadecylindocarbocyanine labeled LDL) (BT-904; Biomedical Technologies, Stoughton, MA) at 5 μg/ml was microfiltered and mixed with phospholipid vesicles (40 μg/ml) and added to the cell medium. Cells were then incubated for an additional 2 hours for Di-HEL uptake. Cells were then fixed and quantified by Cellomics (Halethorpe, MD) Array-Scan essentially as described for the PCSK9-AF647 assay above.

LDLR protein levels were immunoassayed by fluorescence-activated cell sorter (FACS) after an overnight incubation of HepG2 cells in LPDS media, after which cells were removed from plates with trypsin (0.05%) and resuspended in 5% LPDS-containing RPMI 1640 media. The cells in suspension received Adnectins in media in the presence of 10 nM PCSK9 and were incubated overnight at 37°C still in suspension. On the following day, the level of LDLR protein on the HepG2 cell surface was assessed by FACS analysis using biotinylated anti-LDLR polyclonal antibody and streptavidin-PE conjugated detection antibody. Curvefits were as described above.

**Enzyme-Linked Immunosorbent Assay and FRET Assays for Free and Total PCSK9 in Blood Plasma.** Enzyme-linked immunosorbent assays (ELISAs) specific for human and cynomolgus PCSK9 that do not detect mouse PCSK9 were developed. The free (unbound) PCSK9 assay employed streptavidin-pretreated 96-well plates coated with 2 μg/ml of biotinylated PCSK9-Adnectin as capturing reagent. Plasma samples frozen once only were diluted as appropriate in ELISA buffer (25 mM Tris, 150 mM NaCl, pH 7.2 with 0.05% Tween-20 and 0.1% bovine serum albumin), added to wells and incubated for 1 hour at 20°C. Wells were then washed and incubated with 5 μg/ml of rabbit polyclonal anti-human PCSK9 IgG (BMS custom antibody produced by Lampire Biologic Laboratories) for...
1 hour, followed by processing for horseradish peroxidase–labeled anti-rabbit IgG with 3,3′,5,5′-tetramethoxybenzidine by standard ELISA methods. The total PCSK9 ELISA assay was conducted similarly as described above except mAb-4H5 (BMS custom antibody produced by Lampire Biologic Laboratories) was incorporated as the capture antibody. The mAb-4H5 binds the C-terminal domain of PCSK9 and when bound to the 96-well plates efficiently captures total PCSK9 (both Adnectin-PCSK9 complex plus free PCSK9). Standard curves were generated using purified recombinant human or cynomolgus PCSK9.

Because the Adnectin binds cynomolgus PCSK9 more weakly than human, dissociation of the complex in cyno plasma was observed using ELISA assay format, therefore a homogeneous FRET-based assay was established to measure free PCSK9 in cyno monkey plasma. Samples were assayed with minimal dilution (typically 1:4 final) to minimize complex dissociation during the homogeneous assay, which was adapted from the procedure described above for the PCSK9:EGFA FRET assay. A similar assay was developed for use with human and transgenic mouse plasma as described in the results.

**Human PCSK9 Transgenic Mouse Models.** Human PCSK9 overexpresser transgenic mouse originally described by Lagace et al. (2006) were obtained from Dr. Jay Horton (University of Texas South Western Medical School, Dallas, TX) and cohorts were bred and genotyped at BMS. Hemizygous transgenic mice were maintained as mixed strain SJL × C57BL/6J and fed a normal chow diet. This is a human PCSK9 cDNA-driven overexpression model that avidly secretes human PCSK9 to achieve high circulating plasma levels (∼500 μg/ml, ∼7 μM in male hemizygous mice), more than 500-fold greater than normal human levels. The very high levels of human PCSK9 strongly reduce mouse liver LDLR protein leading to hypercholesterolemia.

A second transgenic mouse model was developed that exhibits normal levels of human PCSK9. These mice express full-length human PCSK9 driven from a chromosomal insertion of a human DNA fragment (∼180,000 base pairs) surrounding the PCSK9 gene (22,000 base pairs). Fluorescent in situ hybridization analysis showed insertion in chromosome 5, distinct from endogenous mouse PCSK9 on chromosome 4, which continues to be expressed in these mice. Both mRNAs are expressed in liver of the transgenic mice and undergo regulation by feeding/fasting similarly (data not shown). Plasma levels of human PCSK9 in homozygous male transgenic mice were typically ∼3–5 nM (similar to endogenous mouse protein). The model exhibited no obvious cholesterol phenotype compared with wild type.

Studies used male mice, 28–35 g weight, for both transgenic strains. For dosing, Adnectins were formulated in sterile PBS solution to minimize complex dissociation during the homogeneous assay, which was adapted from the procedure described above for the PCSK9:EGFA FRET assay. A similar assay was developed for use with human and transgenic mouse plasma as described in the results.

**Cynomolgus Monkey Studies.** Vivarium management and the in vivo dosing and sampling protocols for the cyno studies were conducted at Shin Nippon Biomedical Laboratories, Ltd (Everett, WA) in compliance with guidelines from the Association for Assessment and Accreditation of Laboratory Animal Care. Female cynomolgus monkeys, body weight 3.0–4.6 kg, age 4–7 years were used. Blood plasma (K2EDTA) and serum for PCSK9 assays, PK, and clinical chemistry assays were taken at the indicated times and quick frozen for analysis with only a single freeze-thaw cycle. BMS-962476 was formulated in sterile PBS solution for single dose intravenous injection into monkeys.

**Liver mRNA Expression Levels.** Extraction and assay by reverse transcription-polymerase chain reaction of liver mRNAs for key sterol pathway genes was conducted as described by Parker et al. (2013).

**Lipoprotein Profile, Serum LDL-C, and Clinical Chemistry Assays.** The plasma lipoprotein profile from transgenic mice was analyzed by size exclusion chromatography using a Superose 6 column essentially as described (Ha and Barter, 1985) with assay of total cholesterol in the resolved fractions. Serum analytes were assayed on a Siemens Advia 1800 Clinical Chemistry System using standardized enzymatic procedures. LDL-cholesterol was assayed by the direct LDL method (Roche Diagnostics, Indianapolis, IN). Other analytes tested were aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase, total bilirubin, blood urea nitrogen, creatinine, total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, glucose, total protein, albumin, globulins, albumin/globulin ratio, calcium, inorganic phosphorus, sodium, potassium, and chloride.

**Pharmacokinetic Measurements.** BMS-962476 and related protein drug levels were measured in cyno plasma using the Mesoscale Discovery technology platform. Biotinylated human PCSK9 was used to capture the Adnectin and detection was via a rabbit antibody directed against PEG (Epitomics, Burlingame, CA) and a sulfo-tagged goat anti-rabbit polyclonal antibody. Noncompartamental analyses were performed using Phoenix WinNonlin 6.3 (Pharsight Corporation, Mountain View, CA) using a plasma model and linear up/log down calculation method.

**Results.**

**PCSK9 Binding Affinity and Kinetics.** Binding of the Adnectin BMS-962476 to human PCSK9, by both immobilized target and solution phase techniques, exhibited subnanomolar K_D values (Table 1). With the use of biolayer interferometry and surface plasmon resonance methods, BMS-962476 (PEGylated form) exhibited only slightly lower affinity and slower on-rate relative to the unconjugated form, suggesting minimal interference from the PEG chains. With the use of the equilibrium solution method (kinetic exclusion assay), subnanomolar binding to human PCSK9 was also seen (Table 1). A progression to ~10-fold tighter binding over the progenitor Adnectin, clone 1459D05, was seen (Tables 1 and 2). Although the progenitor Adnectin did not detectably bind cyno PCSK9, BMS-962476 bound to cyno PCSK9 with moderate affinity, attributed mainly to faster intrinsic off-rates compared with human PCSK9 (Table 2). Furthermore, no appreciable specific binding of the Adnectin to mouse recombinant PCSK9 was observed by SPR, suggesting that

| Table 1
| --- |
| Affinity of binding to human PCSK9 protein assessed by three different biophysical methods at 25°C, pH 7.4
| Data are mean ± S.D., for n replicates.
<table>
<thead>
<tr>
<th>Form of Adnectin</th>
<th>Biolayer Interferometry (Octet Red) K_D (nM)</th>
<th>Surface Plasmon Resonance (ProteOn) K_D (nM)</th>
<th>Equilibrium Solution Affinity (KinExA) K_D (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1459D05 (progenitor)</td>
<td>3.6 (n = 6)</td>
<td>1.58 ± 0.18 (n = 3)</td>
<td>N.D.</td>
</tr>
<tr>
<td>BMS-962476, his6, no PEG</td>
<td>0.36 (n = 6)</td>
<td>0.29 ± 0.01 (n = 2)</td>
<td>0.07 (n = 2)</td>
</tr>
<tr>
<td>BMS-962476 his6, PEGylated</td>
<td>N.D.</td>
<td>0.65</td>
<td>N.D.</td>
</tr>
<tr>
<td>BMS-962476</td>
<td>N.D.</td>
<td>0.85 ± 0.3 (n = 3)</td>
<td>0.22 (n = 2)</td>
</tr>
</tbody>
</table>

KinExA, kinetic exclusion assay; N.D., not determined.
TABLE 2

Kinetics of binding to human or cynomolgus PCSK9 by surface plasmon resonance at 25°C, pH 7.4

<table>
<thead>
<tr>
<th>Form of Adnectin</th>
<th>$K_d$</th>
<th>$K_{on}$</th>
<th>$K_{off}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PCSK9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1459D05 (progenitor)</td>
<td>1.58 ± 0.18 (n = 3)</td>
<td>1.13E+05</td>
<td>1.80E-4</td>
</tr>
<tr>
<td>BMS-962476, his6, no PEG</td>
<td>0.29 ± 0.01 (n = 2)</td>
<td>2.42E+05</td>
<td>7.03E-05</td>
</tr>
<tr>
<td>BMS-962476, his6, PEGylated</td>
<td>0.65</td>
<td>1.23E+05</td>
<td>8.03E-05</td>
</tr>
<tr>
<td>BMS-962476</td>
<td>0.85 ± 0.3 (n = 3)</td>
<td>6.86E+04</td>
<td>5.61E-05</td>
</tr>
<tr>
<td>Cynomolgus PCSK9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMS-962476, his6, no PEG</td>
<td>8.1</td>
<td>2.49E+05</td>
<td>1.77E-03</td>
</tr>
<tr>
<td>BMS-962476</td>
<td>18.2</td>
<td>2.25E+05</td>
<td>4.11E-03</td>
</tr>
</tbody>
</table>

Data are mean ± S.D., for n replicates.

rodent models could not be employed for functional studies. However, the moderate affinity for cyno PCSK9 was sufficient to support target engagement and pharmacodynamic studies in nonhuman primates.

Using ITC to study the thermodynamics and stoichiometry of the interaction of BMS-962476 with human PCSK9 showed that the equilibrium binding constant ($K_D$) was too tight to measure directly at 25°C (temperature used in the methods employed in Table 1). Measuring ITC at 37°C gave an average $K_D$ of 1.3 ± 0.2 nM, and a van’t Hoff–extrapolated $K_D$ value of 0.24 nM human PCSK9 at 25°C was obtained, agreeing well with the methods above. The change in enthalpy observed ($\Delta H = -29.9 ± 0.1$ kcal/mol) suggested structural stabilization of the complex via specific bond formation at the expense of entropic losses ($-T\Delta S = +17.4$ kcal/mol at 37°C). Furthermore, the ITC results indicated unimolecular binding with stoichiometry approaching 1. Similar ITC results were also observed for the non-PEGylated version of the Adnectin.

The ability of BMS-962476 to bind PCSK9 in undiluted human plasma was assayed using ELISA and homogeneous time-resolved fluorescence/FRET-based methods to measure free PCSK9 after equilibrating samples with the Adnectins. BMS-962476 fully titrated PCSK9 target in plasma with $E_{50}$ values very close to measured levels of PCSK9 in the samples of normal plasma (Fig. 1A) or samples spiked with recombinant PCSK9 (Fig. 1B) to mimic hypercholesterolemic or nant PCSK9 (Fig. 1B) over a surface area of ~910 Å², with contacts coming primarily from residues in the FG loop and N terminus of PCSK9; Adnectin residue numbering is based on Ramamurthy et al. (2012). The FG loop conformation is stabilized by multiple interactions with the Adnectin’s R35, with a β-sheet interaction between Y83 and PCSK9’s S383, hydrophobic contacts from Y83 and H85, and charge–charge interactions between R86 and PCSK9’s E366 and D367. The positively charged N terminus of Adnectin provides favorable interactions with PCSK9’s D374, site of the D374Y gain-of-function mutation. Furthermore, Adnectin BC loop residue Y29 contacts PCSK9 residues 218–221. Among the residues that are in contact with PCSK9, the sequences of Adnectins 1459D05 and BMS-962476 have only a few conservative changes (Y77F, Y79W, and S81A); therefore the bound structure of BMS-962476 is expected to

Fig. 1. Adnectin binding to PCSK9 in human plasma assayed by ELISA and homogeneous time-resolved fluorescence (HTRF). (A) Decrease in free PCSK9 by ELISA in human plasma after addition of the three forms of the PCSK9 Adnectin BMS-962476 as indicated. (B) Decrease in free PCSK9 assayed by HTRF in human plasma after addition of the PCSK9 Adnectins. Open symbols, untreated human plasma; closed symbols, plasma spiked with recombinant human PCSK9 protein. Data are averages of duplicate assays; $E_{50}$ values are in parentheses.
be closely similar to the 1459D05 complex. Near the binding interface (Fig. 2C), the structure of PCSK9 differs from previously described structures in that PCSK9 loop residues 212–218 are visible in one of the molecules in the asymmetric unit; this loop is partially folded away from the catalytic site, burying F216 at a hydrophobic patch on the prodomain from the 2nd molecule in the asymmetric unit (patch formed by prodomain residues A95, I111, V114). A superposition of the 1459D05/PCSK9 crystal structure with those of PCSK9 bound to LDLR (Fig. 2D) indicates that the Adnectin and the EGFA plus EGFB domains cannot simultaneously bind to PCSK9 because of steric overlap.

**LDLR EGFA Competitive Inhibition.** The use of a homogeneous solution phase time-resolved FRET method, the Adnectin competitively displaced binding of the LDLR EGFA peptide to PCSK9 (PCSK9:EGFA binding \( K_D \approx 1 \mu M \)). Potent inhibition of human PCSK9 by BMS-962476 was observed with \( IC_{50} = 2.0 \) nM and maximal inhibition >99% (Fig. 3), consistent with the SPR binding data. Although mouse and Guinea pig PCSK9 bound to EGFA in the FRET assay, little or no inhibition by BMS-962476 was seen (Fig. 3).

**Structural Basis for Species Selectivity.** Examination across species of the amino acid residues on the surface of human PCSK9 that contact the bound Adnectin in the cocrystal structure revealed the basis for the species-selective binding. Among the 37 amino acid residues on human PCSK9 comprising the Adnectin binding site in the three-dimensional structure, only one residue (Q382R) is different between cyno and human PCSK9 (Fig. 4). Guinea pig PCSK9 shares this plus a second residue difference from human (K258R), whereas mouse and rat PCSK9 each have two distinct residue differences (E366K and V380M) compared with human PCSK9 (Fig. 4).

**Inhibition of PCSK9 in Cell-Based Assays.** A high-content, HepG2 cell-based fluorescent LDL (DiI-LDL) uptake assay was developed to measure changes in endogenous.
LDLR functional activity, where inhibition of PCSK9 increases the magnitude of the intracellular LDL fluorescence signal. The assay required relatively high levels of exogenous PCSK9 to promote LDLR degradation (e.g., 135 nM = 10 µg/ml) consistent with previous reports (Benjannet et al., 2010), thus requiring correspondingly higher Adnectin levels for inhibition. Under these conditions in which added PCSK9 decreased LDLR activity ~2.5-fold in HepG2 cells, BMS-962476 fully restored LDLR activity with EC50 ∼31 nM and full inhibition at Adnectin levels approaching 1:1 stoichiometry with PCSK9 concentration (Fig. 5A). The non-PEGylated form showed similar potency (not shown). As an alternative measure of functional activity, internalization of fluorescent PCSK9-AF647 correlated closely with DiI-LDL uptake in the HepG2 cells (Fig. 5, B and C), indicating that PCSK9 and LDLR cointernalized to the endosomal compartment under these conditions. In this assay, the IC50 values for BMS-962476 and its non-PEGylated form were 22 and 33 nM, respectively, with 25 nM PCSK9-AF647 (Fig. 5D). Finally, inhibition of DiI-LDL uptake in HepG2 cells stably transfected with PCSK9-D374Y, the gain of function mutant, showed a similar inhibition profile as exogenous PCSK9-treated parental HepG2 cells (Fig. 5E).

With the use of a FACS-based LDLR depletion assay with added PCSK9 (10 nM), BMS-962476 potently inhibited human PCSK9 as seen by an increase in cell surface LDLR protein (IC50 = 9.7 nM; not shown). Cynomolgus PCSK9 was fully inhibited but with moderate potency (IC50 ∼670 nM) in the FACS-based LDLR assay. Overall the data indicate that BMS-962476 blocks PCSK9 biologic activity by preventing binding and cointernalization with LDLR during endocytosis, resulting in interruption of the subsequent sorting/degradation steps and increased receptor recycling and LDL uptake.

Activity In Vivo in Transgenic Mice. BMS-962476 pharmacology was studied in human PCSK9 transgenic mice because the Adnectin showed no appreciable binding to rodent PCSK9. The overexpressor transgenic mouse described by Lagace et al. (2006) has a strong cholesterol phenotype and provided the first approach to demonstrating in vivo activity of the Adnectin. High dosing levels were required to accommodate the plasma human PCSK9 concentration (∼500-fold higher than humans). Within 3 hours after a single intraperitoneal injection of BMS-962476, plasma total cholesterol fell significantly by up to ∼35% (control average, 285 mg/dl at 0 hour), followed by return to near baseline by 48 hours (Fig. 6A). The lipoprotein profile by size exclusion chromatography

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Fig. 3. Inhibition potency for displacement of EGFA binding to different species of recombinant PCSK9. The graph depicts average values of duplicate assays with BMS-962476 tested against human, cyno, Guinea pig, and mouse recombinant PCSK9 protein. The table lists IC50 values as average ± S.D. (n = 3–5 determinations) in the EGFA FRET assay.

<table>
<thead>
<tr>
<th>Form of Adnectin</th>
<th>Human PCSK9</th>
<th>Cynomolgus PCSK9</th>
<th>Mouse PCSK9</th>
<th>Guinea pig PCSK9</th>
</tr>
</thead>
<tbody>
<tr>
<td>his6</td>
<td>2.6 ± 1.2</td>
<td>19.9 ± 11.2</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>his6 PEG</td>
<td>2.1 ± 1.1</td>
<td>19.0 ± 14.6</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>BMS-962476</td>
<td>2.0 ± 0.6</td>
<td>39.2 ± 28.7</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

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Fig. 4. Amino acid differences among species in the PCSK9 Adnectin binding site. PCSK9 prodomain and catalytic domain showing footprint of Adnectin as lighter colored surfaces (light orange and pale cyan, respectively). Amino acid residues in the PCSK9 binding site that differ among human, cyno, guinea pig, and mouse/rat PCSK9 are highlighted with colors as specified in the table. All other coloring is as in Fig. 2.
showed reductions in the very LDL and LDL peaks in the Adnectin-treated group at 3 to 6 hours postdose (Fig. 6B). The decrease in high-density lipoprotein (HDL) is likely due to particles containing apolipoprotein E (apoE), which is a ligand for the LDLR. The level of free human PCSK9 in plasma was rapidly decreased to near zero (below limit of quantitation) then returned to baseline (Fig. 6C). These findings indicate that the Adnectin rapidly bound circulating PCSK9 and lowered concentrations of plasma apoB- and apoE-containing lipoproteins, consistent with upregulation of liver LDLR activity.

To examine effects of BMS-962476 in a model with more typical levels of PCSK9, a human genomic DNA PCSK9 transgenic mouse model was developed. These mice exhibit plasma human PCSK9 levels around 5 nM and do not have an elevated cholesterol phenotype. With the use of an ELISA specific for human PCSK9, free and total PCSK9 levels were assayed after administration of BMS-962476 as single intraperitoneal doses at low doses (0.1 and 1.0 mg/kg). Strong decreases in free human PCSK9 levels were observed through 48 hours with return to control by day 6 (Fig. 7A). An increase in total human PCSK9 ∼2-fold over controls was also seen over this time course (Fig. 7B), suggesting decreased clearance of the bound Adnectin:PCSK9 complex and continued liver secretion of PCSK9. In a low-dose response study, BMS-962476 inhibited plasma free PCSK9 levels with 50% inhibition at doses as low as 0.01 mg/kg (Fig. 7C).

**Pharmacokinetics in Cynomolgus Monkeys.** Following a single intravenous dose of BMS-962476-his6 or BMS-962476 to monkeys (1 and 5 mg/kg or 5 mg/kg, respectively), BMS-962476 plasma concentrations exhibited a biexponential decline with dose proportional pharmacokinetics. The clearance
was 0.6 to 0.8 ml/h/kg, and the half-life ranged from 74 to 108 hours. The volume of distribution after intravenous administration was 73 to 86 ml/kg, greater than the monkey plasma volumes but less than the volume of extracellular fluid (Brown et al., 1997), suggesting that BMS-962476 largely resides in the extracellular space. BMS-962476 exhibited 79% subcutaneous bioavailability (5 mg/kg dose), indicating that BMS-962476 is likely to be well absorbed in humans after subcutaneous administration.

The elimination mechanisms of BMS-962476 require further investigation, although preliminary data suggest phagocytic uptake processes. PEG-containing vacuolated macrophages and epithelial cells have been observed in some tissues after multiple high doses of BMS-962476 in monkey safety studies (data not shown). This observation is consistent with PEG accumulation, as reported for PEG and for other PEGylated therapeutic proteins (Bendele et al., 1998; Rudmann et al., 2013). In both 2-week and 3-month multiple-dose cyno toxicology studies with BMS-962476, no other findings were noted other than LDL and PCSK9 modulation.

**Pharmacodynamics in Cynomolgus Monkeys.** Following intravenous administration of BMS-962476 to normal cynomolgus monkeys, plasma free PCSK9 levels dropped to near zero (below quantitation limit) by 10 minutes after intravenous administration. Plasma free PCSK9 remained markedly suppressed by over 95% for 48 hours and over 80% for 4 days, then trended back toward baseline (Fig. 8A). The duration of effect was exposure-dependent, with faster return to baseline at the lower dose. These results are consistent with the half-life of the PEGylated Adnectin of ~100 hours, and reflect a return of free PCSK9 to baseline when drug levels reach approximately equimolar levels and no longer effectively titrate PCSK9. Concomitant with free PCSK9 suppression, rapid dose-dependent lowering of LDL-C was seen, reaching an average of 51% below baseline by 48 hours at 5 mg/kg (Fig. 8B). Other than decreased total cholesterol, no effects were seen in 20 other serum clinical chemistry endpoints measured, including HDL cholesterol (see Materials and Methods). LDL cholesterol levels remained below control through 2 weeks, gradually returning to baseline by the end of the 3rd week (Fig. 8B). Plasma total PCSK9 levels increased above baseline by day 3 and continued to rise through day 10, reaching average values ~6- to 7-fold over baseline (~27 nM versus predose ~4 nM). Total PCSK9 levels then converged toward baseline along with free PCSK9 and LDL by day 21 (Fig. 8C). No rebound effect of increasing PCSK9 or LDL-C levels above baseline were observed after drug clearance, suggesting that PCSK9 synthesis or secretion was not upregulated. These findings suggest that blocking PCSK9 function by preventing the LDLR interaction in vivo not only lowers LDL-C by upregulating the receptor but also delays normal clearance of PCSK9 from circulation via LDLR endocytosis in the liver. Thus, the PCSK9-Adnectin complex appears to be cleared at a slower rate than the free form.

In a separate study in cyno monkeys treated with repeat doses of BMS-962476, LDL-C levels were suppressed more
than 70% over 2 weeks (not shown). Similar profiles for free and total PCSK9 were observed as were seen in the single-dose study. No rebound effect on LDL-C levels was observed, with gradual return to baseline after cessation of dosing with no overshoot (not shown), suggesting minimal risk of tachyphylaxis in a clinical setting. At the 2-week point, liver mRNA samples were obtained and expression of key sterol response genes was assayed by reverse transcription-polymerase chain reaction. The liver mRNA concentration for LDLR, PCSK9, HMGCa reductase, and HMGCa synthase as well as sterol regulatory element binding protein 2 (SREBP2) were each suppressed 50–60% versus controls. The liver mRNA concentrations for all 5 genes returned to control levels in cyno monkeys after cessation of dosing for 4 weeks. These data suggest a role for internalized LDL cholesterol contributing to the regulatory pool of sterols, which modulate expression of sterol response genes. The liver mRNA concentration for LDLR, SREBP2, and positions the Adnectin to sterically hinder interactions (e.g., with desolvation, in this case R382 could find favorable compensatory interactions). Binding to this site as the Adnectin, the antibody has a slightly larger footprint (~1150 Å²) and different orientation. The amino acid identity is low between the antibody versus Adnectin residues in contact with PCSK9. Whether due to the natures of the core molecules or the selection processes, the Adnectin and antibody present strikingly different details in their interfacial structure.

The Adnectin core structure here closely resembles the original X-ray crystallography of 10Fn3 itself (Leahy et al., 1996) and previous examples of Adnectins and other 10Fn3-based proteins (Wojcik et al., 2010; Gilbreth et al., 2011; Ramamurthy et al., 2012). As noted for those structures, the primary conformational variations are in the Adnectin BC loop and positions the Adnectin to sterically hinder interactions primarily with the EGFA domain of LDLR. The similar potency of PEGylated and non-PEG forms indicates that the PK-enhancing moiety plays no direct role in competitive inhibition. Unlike some cases where protein or peptide PEGylation reduces affinity (Kubetzko et al., 2005), the Adnectin geometry presents PEG away from the target site with only a minor decrease in binding on-rate.

The cocrystal X-ray structure reported here is the first with more than one PCSK9 molecule in the asymmetric unit and reveals features not typically seen (e.g., PDB IDs 3P5B and 3P5C; Lo Surdo et al., 2011). The usually unresolved segment near the catalytic site (PCSK9 residues 212–218) interacts with the second prodomain in the asymmetric unit and with the bound Adnectin. In one other crystal structure where this loop is resolved, it clasps an antibody with a different conformation (Chan et al., 2009; PDB ID 3H42). In our structure this loop is partially folded away from the catalytic site, burying F216 at a hydrophobic patch on the PCSK9 prodomain from the 2nd molecule in the asymmetric unit (residues A95, I111, V114). These observations suggest that this flexible section of PCSK9 (residues 212–221) could play a role in stabilizing biologically relevant interactions near the catalytic site, for example during autocatalytic self-processing before secretion of PCSK9.

The observed PCSK9 animal species selectivity suggested that amino acid sequence differences in the binding site influenced Adnectin interactions. Overall amino acid homology between human and other species is high, e.g., cyno has 97% identity, whereas mouse, rat, and Guinea pig PCSK9 each have 76% identity and 83–85% similarity. Mapping the species variant residues onto the structure revealed the causes of selective binding. Of 37 amino acids on human PCSK9 covered by Adnectin binding, only one is different in cyno PCSK9 (Q382R). Guinea pig shares this difference plus a second residue (K258R), whereas mouse and rat PCSK9 each have two distinct residue differences compared with human PCSK9 (E366K and V380M). Q382R (cyto) introduces a positively charged residue at the Adnectin-PCSK9 interface. Although burying a charged residue generally reduces binding due to desolvation, in this case R382 could find favorable compensating interactions (e.g., with E76), which could explain the moderate reduction in binding affinity for cyno. K258R (Guinea pig) appears to destabilize the association by removing favorable interactions.

Discussion

Given the challenges to conventional small molecule inhibitor approaches to PCSK9-directed therapeutics, we generated an optimized, high-affinity PCSK9-binding Adnectin as a potential alternative to monoclonal antibodies. Structural analysis of the cocrystal complex of PCSK9 with the progenitor Adnectin 1459D05 showed a binding epitope adjacent to the key LDLR interacting site. Binding to this site covers 37 residues comprising ~910 Å² of PCSK9 surface area and positions the Adnectin to sterically hinder interactions primarily with the EGFA domain of LDLR. The similar potency of PEGylated and non-PEG forms indicates that the PK-enhancing moiety plays no direct role in competitive inhibition. Unlike some cases where protein or peptide PEGylation reduces affinity (Kubetzko et al., 2005), the Adnectin geometry presents PEG away from the target site with only a minor decrease in binding on-rate.

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The Adnectin core structure here closely resembles the original X-ray crystallography of 10Fn3 itself (Leahy et al., 1996) and previous examples of Adnectins and other 10Fn3-based proteins (Wojcik et al., 2010; Gilbreth et al., 2011; Ramamurthy et al., 2012). As noted for those structures, the primary conformational variations are in the Adnectin BC and FG loops and N terminus, which here provide key contacts with PCSK9. The Adnectin N-terminal residues also interact with both PCSK9 and the Adnectin core, stabilizing the competitive binding orientation.

The observed PCSK9 animal species selectivity suggested that amino acid sequence differences in the binding site influenced Adnectin interactions. Overall amino acid homology between human and other species is high, e.g., cyno has 97% identity, whereas mouse, rat, and Guinea pig PCSK9 each have 76% identity and 83–85% similarity. Mapping the species variant residues onto the structure revealed the causes of selective binding. Of 37 amino acids on human PCSK9 covered by Adnectin binding, only one is different in cyno PCSK9 (Q382R). Guinea pig shares this difference plus a second residue (K258R), whereas mouse and rat PCSK9 each have two distinct residue differences compared with human PCSK9 (E366K and V380M). Q382R (cyto) introduces a positively charged residue at the Adnectin-PCSK9 interface. Although burying a charged residue generally reduces binding due to desolvation, in this case R382 could find favorable compensating interactions (e.g., with E76), which could explain the moderate reduction in binding affinity for cyno. K258R (Guinea pig) appears to destabilize the association by removing favorable interactions.
packing of the aliphatic portion of K258 against Y79. E366K (mouse and rat) would eliminate the favorable charge-charge interaction with Adnectin’s R86, and V380M (mouse and rat) may diminish complementary with Y84. These explanations are based on the crystal structure of 1459D05 with PCSK9; however, the overall binding mode for BMS-962476 is expected to be similar with only three conservative changes in PCSK9-contacting residues (Y77F, Y79W, S81A in the Adnectin, which do not contact the species-variant residues).

The species-dependent binding profile led us to study human PCSK9 transgenic mouse models for in vivo pharmacology. The overexpresser human PCSK9 transgenic mouse described by Lagace et al. (2006) provided a strongly hypercholesterolemic model driven by very high circulating levels of human PCSK9. When administered at proportionately high dosages, the Adnectin quickly (3 hours) reduced plasma total cholesterol in concert with free human PCSK9 in plasma, and both returned to baseline 2–3 days later, further illustrating the dynamic physiologic response to PCSK9 modulation.

To study the pharmacodynamics in a preclinical model with normal human PCSK9 levels, a genomic DNA human PCSK9 transgenic mouse model was developed. These mice express full-length human PCSK9 driven from the insertion of a human DNA fragment surrounding the PCSK9 gene, including promoter elements. In homozygous transgenic mice, this resulted in human PCSK9 plasma levels of ~5 nM, insufficient to cause a cholesterol phenotype but allowing binding studies. Here BMS-962476 was very potent with ED50 to cause a cholesterol phenotype but allowing binding studies.

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as a potent and efficacious PCSK9 inhibitor that may have therapeutic potential for LDL lowering in patients with hypercholesterolemia in the treatment of cardiovascular diseases.

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Authorship Contributions

Participated in research design: Mitchell, Chao, Khan, Parker. Conducted experiments: Lo, Monshizadegan, Meyers, Low, Russo, DiBella, Denhez, Myers, Ho, Miao, Khan. Contributed new reagents or analytic tools: Low, Gao, Myers, Duke, Witmer, Miao. Performed data analysis: Mitchell, Chao, Sitkoff, Khan, Parker. Wrote or contributed to the writing of the manuscript: Mitchell, Sitkoff, Parker.

References
