Design and characterization of MP0250, a tri-specific anti-HGF/anti-VEGF DARPin® drug candidate

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ABSTRACT

MP0250 is a multi-domain drug candidate currently being tested in clinical trials for the treatment of cancer. It comprises one anti-vascular endothelial growth factor-A (VEGF-A), one anti-hepatocyte growth factor (HGF), and two anti-human serum albumin (HSA) DARPin® domains within a single polypeptide chain. While there is first clinical validation of a single-domain DARPin® drug candidate, little is known about DARPin® drug candidates comprising multiple domains. Here, we show that MP0250 can be expressed at 15 g/L in soluble form in E. coli high cell-density fermentation, it is stable in soluble/frozen formulation for 2 years as assessed by reverse phase HPLC, it has picomolar potency in inhibiting VEGF-A and HGF in ELISA and cellular assays, and its domains are simultaneously active as shown by surface plasmon resonance. The inclusion of HSA-binding DARPin® domains leads to a favorable pharmacokinetic profile in mouse and cynomolgus monkey, with terminal half-lives of ~30 hours in mouse and ~5 days in cynomolgus monkey. MP0250 is thus a highly potent drug candidate that could be particularly useful in oncology. Beyond MP0250, the properties of MP0250 indicate that multi-domain DARPin® proteins can be valuable next-generation drug candidates.

Introduetion

The quest for more efficacious and safe cancer drug candidates has resulted in the establishment of non-immunoglobulin binding proteins as sources for novel drugs.1 Several drug candidates representing this class of molecules are either approved or at various stages of clinical development.2 The most advanced candidates are single-domain drugs clinically validating the diverse technology platforms. DARPin® domains are a promising class of non-immunoglobulin binding proteins.3 The most advanced DARPin® drug candidate is abicipar pegol, which is being evaluated in Phase 3 clinical trials as a treatment for macular degeneration (ClinicalTrials.gov Identifiers NCT02462486, NCT02462928). DARPin® domains are bio-physically stable molecules that can be expressed at high levels in bacteria, and we have generated numerous high-affinity binding proteins with high target specificity. These findings and the fact that natural ankyrin repeat proteins are often found as multi-domain constructs suggest that the DARPin® platform is ideally suited for the generation of multi-functional molecules. Such approaches have the potential to overcome some of the technical limitations of antibodies and antibody fragments, enabling novel therapeutic strategies.

MP0250, a multi-domain DARPin® drug candidate with binding specificities for vascular endothelial growth factor A (VEGF-A),4 hepatocyte growth factor (HGF),5 and human serum albumin (HSA) is the first multi-functional DARPin® drug candidate in clinical studies. MP0250 is currently being evaluated in a Phase 1 study (ClinicalTrials.gov Identifier NCT02194426) in cancer patients. While the use of VEGF-A antagonists, especially the monoclonal antibody (mAb) bevacizumab, is successful in the clinic, the occurrence of resistance is a major drawback, which preclinical data suggests may be overcome by concomitant inhibition of additional pathways.6–8 With its target specificities, MP0250 may thus help to overcome this problem. Concomitant HGF inhibition appears to be an attractive option, since HGF/cMet signaling has been shown to trigger potent angiogenic signals promoting resistance to anti-angiogenic therapy in some cancer types.9 Cabozantinib, an orally dosed multi-specific tyrosine kinase inhibitor drug that interferes with both the VEGF-A and the HGF/cMet signaling pathways validates this approach.10–12 The drawback of broad specificity drugs (e.g., cabozantinib) and drugs with off-target toxicity (e.g., tivantinib)13,14 is a high incidence of adverse events.15,16 Interestingly, the anti-HGF mAb rilotumumab, which exhibits partial HGF inhibition,17 did not demonstrate efficacy as a single agent in Phase 2 trials in prostate cancer18,19 and in Phase 3 trials in gastric cancer combined with chemotherapy.20 Similarly, onartuzumab,21,22 an anti-cMet antibody, failed to show benefit when tested in combination with the EGFR inhibitor erlotinib in a Phase 3 non-small-cell lung

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cancer trial,\textsuperscript{23} which might be due to inappropriate patient selection.\textsuperscript{23, 24} Creating a treatment option that combines specific HGF/cMet pathway inhibition with concomitant VEGF/VEGF-R2 pathway inhibition thus remains desirable. MP0250 was designed to this end. Here, we describe the generation and characterization of MP0250.

Results

MP0250 consists of an N-terminal DARPin\textsuperscript{®} domain binding HSA, followed by a DARPin\textsuperscript{®} domain binding HGF, followed by a DARPin\textsuperscript{®} domain binding VEGF-A, followed by a C-terminal DARPin\textsuperscript{®} domain binding HSA, all linked by Pro/Thr-rich linkers (Figure 1). Here, we describe MP0250 in detail regarding recombinant expression, solubility, storage stability, potency, pre-clinical pharmacokinetic as well as pharmacological properties. Additional data, in particular data on efficacy in tumor animal models, are given elsewhere.\textsuperscript{25}

High-level recombinant expression

MP0250 can be expressed at high levels in soluble form in the cytoplasm of \textit{E. coli}. Using standard shake-flask expression, 680 mg MP0250 can be expressed in \textit{E. coli} BL21 per liter TB medium culture as judged by SDS-PAGE (Table 1). This yield is in the range of that seen for the individual DARPin\textsuperscript{®} domains, whose expression varied between 320 mg/L and 700 mg/L in the same expression system. These findings indicate that there is no reduction of the expression level when going from a single DARPin\textsuperscript{®} domain to a four-domain construct. Furthermore, the expression levels of the individual DARPin\textsuperscript{®} proteins are significantly higher than the previously reported 200 mg/L for single DARPin\textsuperscript{®} domains,\textsuperscript{26} which were obtained with \textit{E. coli} XL-1 blue and LB medium, indicating significant improvement in yield can be achieved by choice of expression strain and medium. Indeed, when using \textit{E. coli} BL21 and TB medium, the molecule E3_5\textsuperscript{26} can be expressed at 370 mg/L. In fermenter fed-batch productions, titers in the range of 15 g MP0250 per liter culture were achieved as judged by SDS-PAGE, corresponding to $\sim$ 23% of the cell dry weight. These expression levels enable the efficient microbial production of MP0250 for clinical applications. MP0250 consistently can be purified to $> 99\%$ homogeneity using standard chromatography methods such as anion-exchange chromatography, as assessed by size-exclusion chromatography (SEC) and SDS-PAGE.

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**Table 1.** Shake-flask expression levels of MP0250 and DARPin\textsuperscript{®} domains (\textit{E. coli} BL21, TB medium).

| Construct | MP0250 | $\alpha$HSA | $\alpha$HGF | $\alpha$VEGF | E3_5 |
|-----------|--------|-------------|-------------|-------------|------|
| Expression level [g/L] | 0.68 | 0.53 | 0.32 | 0.70 | 0.37 |

\textsuperscript{1}$^\text{GenBank Accession AAO25689.126}$

**Storage stability and solubility**

MP0250, formulated in phosphate-buffered saline (PBS), was assessed at various concentrations with no visible alteration of the sample. In a preliminary long-term stability study at 15 mg/mL in PBS at 25°C, 5°C, $-20\,$°C, or $-70\,$°C, assessed by reverse-phase HPLC (Figure 2), MP0250 exhibited less than 1% loss of monomer peak over at least 18 months at 5°C (0.2% loss at 18 months), $-20\,$°C (0.0% loss at 18 months), or $-70\,$°C (0.0% loss at 18 months). Under accelerated conditions at 25°C, MP0250 exhibited 2.4% loss of monomer peak over a 6-month period. These results demonstrate the commercial feasibility of a liquid MP0250 formulation. Further analyses showed that MP0250 at 15 mg/mL in PBS sustains at least 5 freeze-thaw-cycles without visible changes in SEC, reverse-phase chromatography, and visual appearance (data not shown). The viscosity of MP0250 in PBS was determined to be 1.26 mPaS, 1.39 mPaS, 1.70 mPaS, and 4.16 mPaS at concentrations of 15 mg/mL, 30 mg/mL, 45 mg/mL, and 95 mg/mL, respectively (data not shown), indicating favorable viscosity properties suitable for liquid use of the drug candidate.

**MP0250 is highly potent in inhibiting VEGF-A and HGF**

By embedding an individual domain in a multi-domain molecule, its activity may be altered. It is thus important that MP0250 exhibits sufficient affinity and potency for each of its functionalities. MP0250 is able to bind VEGF-A with an apparent EC\textsubscript{50} of 24 pM (Figure 3 and Table 2). It binds VEGF-A of
human, mouse, and cynomolgus monkey, whereas it does not bind to the related VEGF-C or PDGF-AB (Figure 3a). In sandwich ELISA, MP0250 showed an IC\textsubscript{50} of 4.5 pM in inhibiting human VEGF-A, which is comparable to the individual VEGF-A-binding DARPin\textsuperscript{a} domain (IC\textsubscript{50} of 13 pM) and the value reported for other high-affinity single domain DARPin\textsuperscript{a} proteins\textsuperscript{27} (data not shown). In cell assays, MP0250 was able to inhibit VEGF-A-induced human umbilical vein endothelial cell (HUVEC) proliferation with an IC\textsubscript{50} of 231 pM, comparable to the individual VEGF-A-binding DARPin\textsuperscript{a} domain (IC\textsubscript{50} of 208 pM; Figure 4), which was statistically not significant (p = 0.79, Student’s t-test). These IC\textsubscript{50} values are most likely underestimates because they were limited by the amount of VEGF-A (190 pM) needed in this cell assay. In a VEGF/VEGF-receptor competition fluorescence resonance energy transfer assay, MP0250 was shown to inhibit the interaction between VEGF-A and VEGF-R2 with an apparent IC\textsubscript{50} of 0.7 nM (data not shown). MP0250 binds human HGF with an apparent EC\textsubscript{50} of 24 pM (Figure 3 and Table 2). HGF of mouse and cynomolgus monkey are likewise bound with high affinity by MP0250 (range of 40 to 45 pM; Table 2). HGF inhibition was additionally shown in an A549 cMet phosphorylation cell assay, where MP0250 exhibits an IC\textsubscript{50} of 310 pM (1 nM HGF used for stimulation; Figure 4), again comparable to the HGF-inhibiting individual DARPin\textsuperscript{a} domain (200 pM, difference statistically non-significant; Figure 4). In summary, these data indicate that individual DARPin\textsuperscript{a} domains can be included in a multi-domain construct without compromising functionality. The apparent EC\textsubscript{50} of the HSA binding of MP0250 is significantly improved (13 pM; Figure 3c and Table 2) compared to that observed for the individual HSA-binding DARPin\textsuperscript{a} domain (437 pM; Table 2), which is expected due to the presence of two HSA-binding domains in MP0250 (avidity effect). Species serum-binding ELISA indicate that MP0250 is able to bind serum albumin of human, mouse, and monkey (Figure 3c), as well as rat and dog (data not shown). Overall, these measurements indicate that MP0250 has the potency required for therapeutic use, and that it can validly be used in preclinical mouse and cynomolgus monkey models.

**MP0250 simultaneously binds all targets**

While it might not be required for clinical efficacy, it is of interest whether MP0250 can simultaneously bind to all of its targets. SPR analyses indicate that HGF, VEGF-A, and HSA can be bound simultaneously by MP0250 (Figure 5). Judging from the serum albumin signal, it is likely that MP0250 binds two HSA molecules and thus that all four DARPin\textsuperscript{a} domains simultaneously bind their targets (Figure 5, trace #1). In SEC coupled with multi-angle static light scattering experiments, the ability of MP0250 to simultaneously bind two HSA molecules could indeed be confirmed (data not shown). These

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**Table 2.** Apparent EC\textsubscript{50} values (and 95% confidence interval) of MP0250 for binding VEGF-A, HGF, and serum albumin of different species.

| Species          | EC\textsubscript{50} [pM] VEGF-A | EC\textsubscript{50} [pM] HGF | EC\textsubscript{50} [pM] SA |
|------------------|--------------------------|--------------------------|--------------------------|
| Human            | 24 (20–27)               | 24 (20–29)               | 13 (10–16)               |
| Mouse            | 21 (19–23)               | 45 (38–52)               | 15 (13–17)               |
| Cynomolgus monkey| 24 (24–27)\textsuperscript{1} | 40 (36–44)               | 17 (13–21)               |

\textsuperscript{1}n.a. not analyzed; 100% sequence identity to human VEGF-A, thus human VEGF-A value listed.

\textsuperscript{*}A single HSA-binding DARPin\textsuperscript{a} domain exhibits an EC\textsubscript{50} of 437 pM in the same assay for HSA.
findings support the notion that individual domains can be included in a multi-domain molecule with little compromise of functionality.

The HSA-binding technology leads to a favorable pharmacokinetic profile of MP0250

DARPin® domains without a serum albumin-binding moiety or polyethylene glycol are known to have very fast clearance. While serum albumin binding for pharmacokinetic engineering is a long-standing approach to improve the pharmacokinetic properties of small proteins, no such drug is on the market currently. The background on serum albumin-binding DARPin® domains is described in detail elsewhere. We applied the approach to MP0250, using two HSA-binding DARPin® domains flanking the other two domains. We analyzed the pharmacokinetic properties of MP0250 in single dose experiments in both mouse and cynomolgus monkey. Concentration-time profiles of MP0250 are shown in Figure 6 and the pharmacokinetic parameters calculated by non-compartmental analyses are given in Table 3. In cynomolgus monkey, the pharmacokinetic properties were analyzed in a broad dose range (1 to 100 mg/kg). While Cmax values increased in a dose-proportional manner, exposure (AUC) in the dose range from 1 to 10 mg/kg increased slightly more than dose-proportional, leading to a decrease in clearance between the low and higher dose levels. Since time points for serum sampling was limited to 168 h (one week), a substantial part of the overall exposure (AUC_inf) was extrapolated, which might have contributed to the observation of a slight non-dose linear behavior. The absence of obvious concentration outlier measurements in both mouse and monkey indicates the absence of anti-drug antibodies, which is in line with expectations for one-week studies. The pharmacokinetics both in mouse and cynomolgus monkey indicate the feasibility of preclinical testing in mouse and cynomolgus monkey. Furthermore, a potential terminal half-life of 11 days in human is extrapolated for MP0250 from mouse and cynomolgus monkey data by allometric scaling. These results indicate a pharmacokinetic profile of MP0250 suitable for therapeutic use as a multi-functional antagonist.
Discussion

Having well-behaved individual domains is an important starting point for achieving a developable multi-domain drug candidate. Here, we show that the advantageous properties seen in single DARPin/C210 domains are retained in the multi-domain DARPin/C210 drug candidate MP0250. For example, it has a high recombinant expression yield, high solubility and high storage stability, enabling successful manufacturing of the drug. Its pharmacokinetic profile and pharmacologic activity make MP0250 a promising candidate for cancer therapy. Fiedler et al. have shown efficacy of MP0250 in a number of mouse tumor xenograft models, indicating that the drug candidate indeed has the potential to improve patient benefit beyond single agent anti-VEGF therapy. Based on the results shown here, the preclinical in vivo data, as well as safety and tolerability assessments, clinical testing of MP0250 has been initiated (ClinicalTrials.gov Identifier NCT02194426).

Materials and methods

Ribosome display and cloning

Individual DARPin® domains binding VEGF-A, HGF, and HSA, respectively, were selected by ribosome display as described in patent applications WO2010060748, WO2014191574, and WO2012069654, respectively. The DARPin® domain amino acid sequences used to build MP0250 and the amino acid sequence of MP0250 are detailed in patent application PCT/EP2016/057272. MP0250 and proteins comprising one or more DARPin® domains were generated using DNA synthesis or by standard cloning methods.

Protein expression and purification

Single domain proteins and multi-domain proteins were expressed and purified as described previously or using standard chromatography methods. For the expression tests, E. coli BL21 transformed with a standard T5 expression plasmid harboring MP0250 or individual DARPin® domains was used with 50 mL TB growth medium in 300 mL shake-flasks at 220 rpm in a Novotron (Infors, Switzerland) shaker at 37°C. Cultures were induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranosid (IPTG) at an OD600 of 1.0 and the cultures were incubated for five more hours. Cells were harvested by centrifugation. Equivalent amounts of cells were analyzed by SDS-PAGE. Proteins were quantified by graphical methods in comparison to defined amounts of individual DARPin® proteins or MP0250. For high cell-density fermentation, E. coli HMS174 transformed with the expression plasmid harboring MP0250 was used with minimal medium using a 5 L bioreactor (Infors Labfors 5), in a fed-batch mode controlling pH at 6.8, temperature and at 37°C, pO2 level above 20%. Product expression was induced by adding 1 mM IPTG per liter at a cell density of 50 g/L dry cell weight. Amounts of product were judged using SDS-PAGE and cell dry weight was determined using standard methods.

Table 3. Pharmacokinetic properties of MP0250 i.v. administered in mouse and cynomolgus monkey.

| Parameter              | Unit | Balb/c mice 1 mg/kg | Balb/c mice 2 mg/kg | Cynomolgus monkey 1 mg/kg | Cynomolgus monkey 10 mg/kg | Cynomolgus monkey 100 mg/kg |
|------------------------|------|---------------------|---------------------|---------------------------|-----------------------------|-----------------------------|
| Number of animals      |      | 10f                 | 10f                 | 5f/5m                      | 5f/5m                       | 5f/5m                       |
| AUCINF_pred            | hr*(nmol/L) | 12901               | 32727               | 43217                      | 643593                      | 6698389                      |
| AUClast                | nmol/L | 11693               | 29309               | 30633                      | 384761                      | 3755348                      |
| Cmax                   | hr    | 0.083               | 0.083               | 0.583                      | 1.375                       | 0.583                        |
| Tmax                   | hr    | 28.5                | 29.7                | 94.6                       | 127.0                       | 138.9                        |
| Vz                     | mL/kg | 51.1                | 41.9                | 52.1                       | 45.9                        | 48.3                         |
| t1/2                   | hr    | 28.5                | 29.7                | 94.6                       | 127.0                       | 138.9                        |

*Averaged all animals per group.*

Figure 6. Concentration-time profiles of MP0250. (a) Mouse pharmacokinetic profile of MP0250 at 1 mg/kg (filled circles) and 2 mg/kg (filled squares). (b) Cynomolgus monkey pharmacokinetic profile of MP0250 at 1 mg/kg (open circles), 10 mg/kg (open squares), and 100 mg/kg (open triangles). Mean concentrations and standard deviations are plotted over time. Pharmacokinetic parameters derived from the experiments are given in Table 3.
For EC50 measurements, 50 μL or 100 μL of 20 nM target (human VEGF-A, mouse VEGF-A, human VEGF-C, human PDGF-AB (all R&D Systems); human (Peprotech), cynomolgus monkey (Sino Biological), and mouse (R&D Systems) HGF) or 100 μL of 1/30’000 diluted serum (human (Sigma), mouse (Innate research), cynomolgus monkey (collected in PBS unless stated otherwise). Storage stability analyses, proteins were concentrated to 300 μL/PBST (PBS supplemented with 0.1% Tween 20), the wells were blocked with 300 μL/well or 50 μL/well MP0250 or individual DARPin® proteins (concentrations ranging from 100 nM to 0.001 μM) and polyclonal anti-human-IgG-d2 (Cisbio) were added to the wells of the plate and the plate was incubated for 1 h protected from light at room temperature. The ratio of fluorescence at 665 nm and 620 nm was measured.

**ELISA, competition ELISA and homogeneous time-resolved fluorescence**

For EC50 measurements, 50 μL or 100 μL of 20 nM target (human VEGF-A, mouse VEGF-A, human VEGF-C, human PDGF-AB (all R&D Systems); human (Peprotech), cynomolgus monkey (Sino Biological), and mouse (R&D Systems) HGF) or 100 μL of 1/30’000 diluted serum (human (Sigma), mouse (Innate research), cynomolgus monkey (collected in PBS unless stated otherwise). Storage stability analyses, proteins were concentrated to 300 μL/PBST (PBS supplemented with 0.1% Tween 20), the wells were blocked with 300 μL/well or 50 μL/well MP0250 or individual DARPin® proteins (concentrations ranging from 100 nM to 0.001 μM) and polyclonal anti-human-IgG-d2 (Cisbio) were added to the wells of the plate and the plate was incubated for 1 h protected from light at room temperature. The ratio of fluorescence at 665 nm and 620 nm was measured.

**Surface plasmon resonance**

SPR measurements were performed using a ProteOn XPR36 instrument (BioRad). Running buffer was PBS pH 7.4 containing 0.005% Tween 20. 3000 RU of HGF were immobilized on a GLC chip using 100 nM HGF (Peprotech, 100–39) in 10 mM sodium acetate buffer pH 5.3. For the analysis, 100 nM MP0250 (180 s association, 60 s dissociation) was first injected, followed by 100 nM VEGF (180 s association, 60 s dissociation; Relia Tech, 30-036-L), and 100 nM serum albumin (180 s association, 300 s dissociation; CSL Behring 100 mL, 200 g/L). Different controls were made by omitting one each of the injected components (see Figure 5). All signals were referenced to the blanks.

**Cellular assays**

Purified MP0250 and individual DARPin® domains were tested in cellular assays, including a HUVEC proliferation assay to assess VEGF-A inhibition, and a cMet phosphorylation assay to assess HGF inhibition. For the HUVEC proliferation assay, human VEGF-A was used at a concentration of 8 ng/mL (corresponding to EC80 as determined in a proliferation assay; ~190 pM), and MP0250 or the individual VEGF-A-inhibiting DARPin® domain of MP0250 were titrated in a concentration range between 15 pM and 2.5 nM. About 3000 cells were seeded in 50 μL EBDM-2 medium (Lonza) supplemented with penicillin/streptomycin and 5% fetal calf serum (assay medium) in CellBind plates (Sigma-Aldrich) and an optical reader (450 nm; 620 nm reference).

Inhibition of cMet phosphorylation by MP0250 was measured using A549 cells and a DuoSet P-cMet-ELISA (RnD Systems). Cells were seeded in complete medium with 10% fetal calf serum in 12-well plates at 200’000 cells per well in complete
medium. After 24 h, medium was replaced by serum-free medium. Cells were incubated for another 24 h and stimulated by 1 nM human HGF (or PBS for negative control) in the presence and absence of MP0250 or the individual HGF-inhibiting DARPin® domain. HGF and MP0250 or the individual DARPin® domain were preincubated for at least 30 minutes at room temperature prior to addition to cells. Cells were stimulated for 10 minutes at room temperature. Stimulation was terminated by removing the cell supernatant, cell washing with PBS, and addition of ice-cold cell lysis buffer (Sample diluent concentrate 2x, DYC002 (RnD Systems) plus phosphatase inhibitor (Roche) plus protease inhibitor (Roche)). Cell lysates were kept at −20°C until the ELISA experiment in a 96-well plate.

**Pharmacokinetic measurements**

Single-dose tail vein intravenously administered dose pharmacokinetic measurements in female Balb/c mice (n = 10 per group) were performed at target doses of 1 mg/kg and 2 mg/kg MP0250. Blood samples were collected pre-dose and again at 5 min and 4, 8, 24, 72, and 96 hours post-injection. Concentrations were determined as described below. The pharmacokinetic profile of MP0250 in cynomolgus monkey was assessed by administering single doses of MP0250 via intravenous infusion for 30 min at target dose levels of 1 mg/kg, 10 mg/kg, and 100 mg/kg to 5 each male monkey was assessed by administering single doses of 1 mg/kg, 10 mg/kg, and 100 mg/kg to 5 each male

**Disclosure of potential conflicts of interest**

All authors hold options or shares in Molecular Partners AG.

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