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Recommended Citation
Berezhnoy, Alexey; Sumrow, Bradley J; Stahl, Kurt; Shah, Kalpana; Liu, Daorong; Li, Jonathan; Hao, Su-Shin; De Costa, Anushka; Kaul, Sanjeev; Bendell, Johanna; Cote, Gregory M; Luke, Jason J; Sanborn, Rachel E; Sharma, Manish R; Chen, Francine; Li, Hua; Diedrich, Gundo; Bonvini, Ezio; and Moore, Paul A, "Development and Preliminary Clinical Activity of PD-1-Guided CTLA-4 Blocking Bispecific DART Molecule." (2020). Articles, Abstracts, and Reports. 4346.
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Authors
Development and Preliminary Clinical Activity of PD-1-Guided CTLA-4 Blocking Bispecific DART Molecule

Graphical Abstract

Highlights
- PD-1 and CTLA-4 are co-expressed by TILs but not healthy lymphocytes
- MGD019 is designed to block PD-1 and deliver enhanced CTLA-4 blockade in TME
- MGD019 is safe in NHP while demonstrating biomarkers of PD-1 and CTLA-4 inhibition
- Encouraging activity in tumors traditionally unresponsive to checkpoint blockade

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In Brief
Co-blockade of PD-1 and CTLA-4 increases benefits in cancer immunotherapy but also its toxicity. Berezhnoy et al. constructed a bispecific DART molecule to deliver safer and potentially more effective co-blockade by targeting CTLA-4 inhibition to tumors. MGD019 demonstrates encouraging activity in tumors traditionally unresponsive to checkpoint blockade.

Berezhnoy et al., 2020, Cell Reports Medicine 1, 100163
December 22, 2020 © 2020
https://doi.org/10.1016/j.xcrm.2020.100163
Article

Development and Preliminary Clinical Activity of PD-1-Guided CTLA-4 Blocking Bispecific DART Molecule

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SUMMARY

Combination immunotherapy with antibodies directed against PD-1 and CTLA-4 shows improved clinical benefit across cancer indications compared to single agents, albeit with increased toxicity. Leveraging the observation that PD-1 and CTLA-4 are co-expressed by tumor-infiltrating lymphocytes, an investigational PD-1 x CTLA-4 bispecific DART molecule, MGD019, is engineered to maximize checkpoint blockade in the tumor microenvironment via enhanced CTLA-4 blockade in a PD-1-binding-dependent manner. In vitro, MGD019 mediates the combinatorial blockade of PD-1 and CTLA-4, confirming dual inhibition via a single molecule. MGD019 is well tolerated in non-human primates, with evidence of both PD-1 and CTLA-4 blockade, including increases in Ki67+CD8 and ICOS+CD4 T cells, respectively. In the ongoing MGD019 first-in-human study enrolling patients with advanced solid tumors (NCT03761017), an analysis undertaken following the dose escalation phase revealed acceptable safety, pharmacodynamic evidence of combinatorial blockade, and objective responses in multiple tumor types typically unresponsive to checkpoint inhibitor therapy.

INTRODUCTION

Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) inhibit a variety of T cell functions, such as proliferation, cytokine production, and cytotoxicity. Induced upon activation, CTLA-4 expression by T cells leads to their functional inhibition by multiple mechanisms, including competition with CD28-mediated activation1 and removal of co-stimulatory ligands from antigen-presenting cells (APCs).2 CTLA-4 is also constitutively expressed by regulatory T cells (Tregs) and is essential to maintain self-tolerance. Knockout of CTLA-4 in Tregs does not affect their survival, but it does eliminate their suppressive function.3 When expressed by T lymphocytes, among other cells, PD-1 acts as an inhibitory molecule that reduces cytotoxicity and cytokine production. Localized expression of programmed death ligand 1 (PD-L1) and PD-L2 is induced in prolonged inflammatory environments, where it protects affected tissues from immune attack4 and preserves chronically activated T cells in a suppressed yet viable state.5 Mechanistic studies revealed that, while both CTLA-4 and PD-1 suppression rely on the CD28 co-stimulation pathway,6 the 2 molecules have separate but complementary roles in T cell regulation.

Both PD-1 and CTLA-4 are co-opted by tumors to enable immune evasion, the escape of otherwise immunosensitive tumors from T cell-mediated lysis.7 Antibody-mediated blockade of CTLA-4 or PD-1 mediates antitumor activity in murine models.7,8 In the clinic, both PD-1- and CTLA-4-targeted therapies have yielded therapeutic benefit, while their combination has demonstrated improved responses in advanced melanoma, non-small cell lung carcinoma (NSCLC), renal cell carcinoma, hepatocellular carcinoma, and microsatellite instability-high colorectal cancer.9–13 Inhibiting PD-1 predominantly re-activates exhausted CD8 T cells14 and their precursors,15 without induction of new antitumor effectors. While PD-1/PD-L1 axis blockade has become a mainstay of cancer therapy,16,17 the magnitude and
durability of its clinical effects strongly depend on preexisting tu-
mor-specific T cell populations and could be further
augmented by the expansion of effector T cells in periphery. CTLA-4 inhibition by the monoclonal antibody (mAb) ipilimumab induces polyclonal T cell activation and expansion, yielding a more diversified antitumor immune response, accompanied by the expansion of inducible T cell co-stimulator (ICOS) CD4 T cells. The complementary pharmacodynamic mechanisms of PD-1 and CTLA-4 blockade underlie the improved clinical efficacy observed upon combinatorial blockade of both check-
points, with ipilimumab plus the anti-PD-1 mAb, nivolumab, resulting in enhanced antitumor activity beyond levels achievable by either blockade alone. Clinical improvements in combina-
tion therapy, however, were accompanied by the increased fre-
quency and severity of treatment-related adverse events (TRAEs). Fifty-five percent of patients receiving the combination of ipilimumab and nivolumab experienced severe (i.e., grade ≥ 3) TRAEs, a significant increase compared to 16% for nivolumab and 27% for ipilimumab when administered alone. Beyond the potential medical consequences, severe TRAEs often neces-
sitate treatment alterations (e.g., reduction of recommended
doses, discontinuation of treatment, immunosuppressive ther-
apy), introducing factors that may limit the therapeutic benefits
of combinatorial blockade in some patients.

Novel strategies, such as targeting immune interventions to
the tumor microenvironment (TME) or Fc effector domain modulation, may increase treatment benefit and reduce im-
munemediated toxicity. In mouse models, the antitumor effects
of anti-CTLA-4 treatments were related to blockade of CTLA-4 in
the TME and tumor-draining lymph nodes, while adverse effects
were associated with the Fc-dependent effector function of anti-
CTLA-4 blockers in healthy tissues.

Agents capable of optimal co-blockade of these two check-
points should preserve PD-1 neutralization in all compartments
together with maximal CTLA-4 inhibition in the TME, without
depletion of Treg in normal tissues. While such differential
blockade is beyond the reach of conventional antibodies, pur-
pose-engineered molecules could provide a solution. To
address this challenge, we engineered a tetravalent bispecific
PD-1 x CTLA-4 molecule (MGD019) by using the bispecific
DART® platform. The molecule was designed to fully block
PD-1 while exerting increased CTLA-4 blockade on dual-ex-
pressing cells, such as tumor-infiltrating lymphocytes (TILs), to
preferentially direct the co-blockade activity to the TME. Built
on an immunoglobulin G4 (IgG4) backbone, MGD019 showed
no Fc-mediated effector function while augmenting human T cell activation in vitro. MGD019 was well tolerated in cynomol-
gus monkeys and yielded preliminary evidence of clinical activity with acceptable safety in heavily pre-treated, advanced solid tu-
mor cancer patients.

RESULTS

Cells Co-expressing PD-1 and CTLA-4 Abound in the TME Compared to Normal Tissues

Dual in situ hybridization (ISH) and multicolor flow cytometry were used to define the expression pattern of PD-1 and CTLA-
4 in tumors, peripheral blood, and healthy tissues (Figure 1).

ISH analyses of an ovarian cancer tumor tissue microarray (TMA) revealed detectable PD-1+ and CTLA-4+ cells at varying
levels in most specimens (Figure 1A). On a cellular basis, CTLA-4 and PD-1 expression was associated primarily with tu-
mor immune infiltrate, with cells co-expressing both molecules readily detected (Figure 1B). In contrast, analysis of a 35-healthy
adult tissue TMA revealed no expression of PD-1 or CTLA-4, with the exception of lymphoid organs (thymus, tonsils, and lymph
nodes) and rare occurrences in the stroma of colon and pancreas (data not shown). Notably, PD-1 and CTLA-4 expres-
sion in normal lymphoid tissues was observed in distinct, spatially separated cell populations, in contrast to the pattern of co-expression observed in TILs (Figure 1B). Digital quantita-
tion confirmed a higher proportion of PD-1/CTLA-4 double-positive cells in ovarian (Figure 1B), breast, lung, colon, and rectal
cancer specimens relative to those observed in normal lymphoid
(Figures 1C and S1). Flow cytometry studies comparing circulating T cells from healthy donors and TILs from patients
with various cancers confirmed cell surface protein expression
(Figures 1D and 1E). On average, 14.6% of TILs expressed
both PD-1 and CTLA-4, 51.8% expressed PD-1 alone, 2.8% ex-
pressed CTLA-4 alone, and 30.8% did not express either
protein. This observation is in line with a prior report of a high
occurrence of PD-1 and CTLA-4 expression on TILs. Interest-
ingly, a small but detectable fraction (<0.25%) of circulating
T cells from tumor patients co-expressed PD-1 and CTLA-4,
while no circulating double-positive cells were detected in
healthy donors (Figures 1D and 1E). These data indicate that
cells co-expressing PD-1 and CTLA-4 are prevalent in the TME
but virtually absent in healthy tissues. This observation further
implies that targeting dual PD-1/CTLA-4 expressing cells
may provide an opportunity for selective checkpoint blockade in
the TME, while relatively reducing effects in normal tissues.

Engineering and Characterization of MGD019, a PD-1 x
CTLA-4 Bispecific Molecule Featuring Complete
Blockade of PD-1 and Variable Inhibition of CTLA-4

To build a molecule capable of stringent, uniform blockade of
PD-1 and conditional blockade of CTLA-4, we selected a high-
affinity, clinically validated anti-PD-1 mAb and an anti-
CTLA-4 mAb with ligand-blocking properties similar to that of
ipilimumab (see Method Details) as a precursor for the PD-1
and CTLA-4 arms, respectively. A PD-1 x CTLA-4 bispecific
molecule was constructed on the DART® platform in a symmet-
tric, tetravalent 2 x 2 format (designated MGD019; Figure 2A),
with a hinge-stabilized IgG4 backbone to limit Fc-dependent
effector functions, including antibody-dependent cell cytotox-
icity (ADCC). The choice of Fc domain was primarily driven by
the desire to limit the potential depletion of PD-1+ activated
T cells and to avoid the adverse effects of Treg depletion.

MGD019 binds cell surface expressed PD-1 and blocks
PD-L1 binding with a potency profile superimposable with that of
its retifanlimab precursor (Figure 2B) or a replica of nivolumab (Figure S2A). When a bivalent 1 x 1 format was used, however,
PD-1 ligand-binding blockade was significantly compromised
(Figure S3), indicating avidity requirements to maintain maximum
PD-1 inhibition on single-positive cells. In contrast to the behavior
of the PD-1 binding arm, CTLA-4-binding and B7.1-binding
blockade were reduced in the MGD019 format relative to the parental mAb (Figure 2C) or a replica of ipilimumab (Figure S2B). However, maximal blockade of B7.1 ligand/CTLA4 interaction on CTLA4-only expressing cells was achieved at higher concentrations. Higher binding saturation levels were observed on PD-1/CTLA-4 double-positive cells, consistent with the ability of MGD019 to interact with PD-1 and CTLA-4 independently of each other (Figure 2E).

A key feature of bispecific molecules is the potential for simultaneous antigen recognition in cis on the same cell, a mechanism that can promote binding cooperativity through avidity. Enzyme complementation following MGD019-mediated co-ligation of PD-1 and CTLA-4 expressing cells was achieved at higher concentrations. Higher binding saturation levels were observed on PD-1/CTLA-4 double-positive cells, consistent with the ability of MGD019 to interact with PD-1 and CTLA-4 independently of each other (Figure 2E).

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Figure 2. MGD019 Molecular Structure and Bispecific Binding to PD-1 and CTLA-4

(A) MGD019 is a tetravalent bispesific (2 × 2) Fc-bearing DART molecule.

(B) Binding of MGD019 (red diamonds), parental PD-1 mAb retifanlimab (blue squares), parental CTLA-4 mAb 4B6 (green triangles), or isotype control (black circles) to Jurkat/PD-1 cells and blockade of PD-L1 binding to the cells.

(C) Binding to Jurkat/CTLA-4 cells and blockade of B7-1 binding to the cells.

(D) Re-activation of β-galactosidase (β-gal) upon co-engagement of PD-1 and CTLA-4 by MGD019 in PathHunter PD-1+CTLA-4+ assay. Error bars depict standard errors of the mean (SEMs).

(E) Binding to in vitro-stimulated, PD-1+/CTLA-4+ primary T cells and blockade of B7-1 binding to Jurkat PD-1+/CTLA-4+ cells.

(F) Blockade B7.1 binding to Jurkat PD-1+/CTLA-4+ by MGD019 or CTLA-4 mAbs alone or in the presence of a 103 concentration of competing PD-1 mAbs (open red diamonds and purple crosses, respectively).

(G) Interaction of MGD019 with single- and dual-expressing cells. Average (EC50) values of PD-1 (blue) and CTLA-4 (yellow) ligand binding blockade (Table S1). Representative experiments out of ≥ 3 independent repeats are shown in (B)–(F).

See also Table S1 and Figures S2 and S3.
assays, with activity again comparable to that of mAb combinations (Figure S4). In ~25% of healthy donors, in whom PD-1 or CTLA-4 blockade with individual blocking mAbs did not substantially affect staphylococcal enterotoxin B (SEB)-driven T cell activation, MGD019 but not the combination of 2 mAbs enhanced interleukin-2 (IL-2) release, although the magnitude of the effect was variable between donors. Unlike ipilimumab, MGD019 did not reduce the number of FoxP3+ cells in vitro (Figures 3C and 3D).

MGD019 Is Well Tolerated in Non-human Primates

The toxicity profile, pharmacokinetic (PK), and pharmacodynamic activities of MGD019 were evaluated in cynomolgus monkeys, a relevant cross-reactive species. MGD019 binding affinity to cynomolgus monkey PD-1 or CTLA-4 is in the range of the human target antigens. Repeat intravenous (i.v.) administrations (4 weekly doses) of MGD019 were well tolerated at dose levels of 10, 40, and 100 mg/kg (Table 1). In-life effects were limited to an increased incidence of soft/watery feces at ≥ 40 mg/kg and minor hematological changes at ≥ 10 mg/kg. Spleen weight parameters were increased compared to controls for males at doses of ≥ 40 mg/kg and females at doses of ≥ 10 mg/kg, which correlated microscopically with generalized lymphoid hyperplasia characterized by increased numbers of lymphocytes affecting all compartments of the lymphoid tissues. All of the effects were reversible following a 10-week recovery period and were not considered adverse. The no-observed-adverse-effect level was 100 mg/kg, the highest dose tested. MGD019 demonstrated linear PK (half-life ~7 days) across the dose range tested (Figure 4A). All animals within the individual dose groups achieved comparable exposure to MGD019 during the first dose interval; however, exposure decreased in some
animals during the fourth dose interval due to the appearance of anti-drug antibodies (ADAs). The level of MGD019 binding to PD-1-expressing circulating T cells correlated with its serum concentration (Figure 4B). A dose-dependent shift in the relative proportion of circulating T cells with a memory-like phenotype at the expense of naive T cells (Figure 4C and S5C), together with an increase in the fraction of splenic ICOS-expressing CD4+ T cells (Figure 4D), were observed in MGD019-treated animals, while no change in tissue-resident or circulating Treg populations were apparent (Figure 4E). These pharmacodynamic changes are consistent with previously reported effects of CTLA-4 blockade in vivo.34,35

To distinguish the effects of dual PD-1 plus CTLA-4 check-point blockade in vivo from that of PD-1 blockade alone, the effects of MGD019 in cynomolgus monkeys were compared to those of its parental PD-1 mAb. Both molecules were associated with evidence of T cell proliferation (Figures 4F and S5B), although MGD019-treated animals had more prominent changes in the Ki67+ T cell fraction and showed T cell expansion in the spleen (Figure S5A), indicating the additional impact of CTLA-4 blockade by the DART molecule.

First-in-Human Study Highlights Clinical Activity and Correlative Pharmacodynamics

Patients with advanced, previously treated solid tumors of any histology were enrolled in a Phase I study of MGD019 at escalating doses of 0.03, 0.1, 0.3, 1, 3, 6, and 10 mg/kg administered as 30-min i.v. infusions every 3 weeks in a 3 + 3 design. Thirty-three patients (median age 61 years; 51.5% male; 3 median prior lines of therapy) representing 21 different advanced solid tumor types were treated before the data cutoff, including 13 patients (39.4%) previously receiving checkpoint inhibitor therapy. MGD019 demonstrated linear kinetics with a half-life of 3 mg/kg maintain serum trough concentrations of MGD019 comparable to those of ipilimumab or nivolumab (Figures 5A and S6A). MGD019 bound circulating T lymphocytes (Figure 5B) occupying and blocking PD-1 for durations proportional to dose and serum concentrations (Figure 5C). MGD019 administration was associated with enhanced proliferation of peripheral CD8+ T cells, but no associated changes in Treg population (Figure 5D). Furthermore, a dose-dependent upregulation of ICOS on circulating CD4+ T cells was observed (Figures 5E and S6C), with the highest frequency and positivity in patients treated at a dose ≥ 3 mg/kg.

MGD019 was generally well tolerated up to the top predefined dose level of 10 mg/kg, with no dose-limiting toxicities (DLTs) observed. As such, the maximum tolerated dose (MTD) was not exceeded or defined. After enrolling additional patients to further explore PK/pharmacodynamic relationships and clinical activity, intolerability at 10 mg/kg became evident. As of the data cutoff of April 1, 2020, TRAEs occurred in 26/33 (78.8%) patients, most commonly fatigue (24%), nausea, arthralgia, pruritus, and rash (18% each). The rate of grade ≥ 3 TRAEs was 24.2% (Table 2; Figure S6D). Among 8 patients treated at 10 mg/kg, notable immune-related adverse events included grade 3 events of myocarditis, enterocolitis, and maculopapular rash. No grade 4 or 5 TRAEs were observed. Table 2 displays TRAEs observed in ≥ 5% of patients, as well as adverse events of special interest (AESI) occurring at lower frequencies.

Treatment-related serious adverse events (SAEs) included enteritis, enterocolitis, pneumonitis, and myocarditis (n = 1 each) and occurred at dose levels ≥ 3 mg/kg; all of the patients recovered without sequelae after discontinuation of MGD019 and appropriate treatment. Infusion-related reactions (IRRs) were observed (n = 5, 15.2%) and were mild to moderate in severity. Among 25 response-evaluable patients, objective responses per Response Evaluation Criteria in Solid Tumors version 1.1 were observed in 4 patients (including 1 unconfirmed response), with tumor types conventionally unresponsive to checkpoint inhibition. These 4 patients were among 13 response-evaluable patients treated at doses ≥ 3 mg/kg (Figure 5G) and each demonstrated ICOS upregulation on circulating CD4+ T cells. Confirmed objective responses occurred in patients with microsatellite-stable colorectal cancer (Figure 5F), metastatic type AB thymoma (both partial responses [PRs]), and metastatic castration-resistant prostate cancer (complete response [CR]) with resolution of elevated pre-treatment prostate-specific antigen. In addition, an unconfirmed partial response was observed in an anti-PD-L1-refractory serous fallopian tube carcinoma patient with a >50% reduction of CA-125. Nine patients had stable disease as a best response.

DISCUSSION

The engineering of a molecule capable of simultaneous blockade of PD-1 and CTLA-4 must be informed by both clinical considerations and biological context. Given the critical clinical role and favorable safety profile that systemic PD-1/PD-L1 axis blockade plays in tumor immunotherapy, an ideal combinatorial compound should not compromise the ability of the molecule to block the interaction between PD-1 and its ligands. In contrast, CTLA-4 blockade may carry greater propensity for untoward effects, which could be limited by conditioning the CTLA-4 arm to
block preferentially in the context of PD-1/CTLA-4 dual-expressing cells present in the TME. MGD019 provides PD-1 blockade in vitro comparable to stand-alone anti-PD-1 molecules (i.e., retilanlimab, its precursor molecule, or nivolumab) and a variable degree of CTLA-4 blockade. In PD-1+/CTLA-4+ cells, MGD019 blockade of CTLA-4 ligand binding is reduced compared to that mediated by anti-CTLA-4 mAbs, while in TIL-like dual-expressing cells, the potency of CTLA-4 blockade can increase by 2 orders of magnitude (Figure 2), in agreement with an avidity-driven effect. Consistent with this observation, dual checkpoint blockade by MGD019 in primary T cell models provided an enhanced effect in a subset of donors selected for poor response to PD-1 blockade in vitro.

MGD019, by virtue of an IgG4 Fc region, has a limited capacity for Fc-mediated ADCC, sparing potential depletion of targeted cells, which would have included effector T cells in addition to Treg cells. The impact of Treg depletion on the efficacy and safety of ipilimumab is still uncertain, enabling the Fc of anti-CTLA-4 agents to mediate depletion, however, has been linked to both activity and adverse effects in mouse models. Avoiding CTLA-4-mediated Treg depletion while maintaining strong CTLA-4 blockade in the TME may contribute to improving safety, while maintaining the efficacy associated with CTLA-4 antagonism. Furthermore, the main immunosuppressive effect of tumor-associated Treg cells can be ascribed to CTLA-4 functional activity. MGD019 blockade of CTLA-4 in the TME may be sufficiently strong to compensate for the absence of Treg depletion due to increased avidity in dual-expressing cells and ability to create a high local concentration of PD-1 anchored CTLA-4 inhibitor.

The PK characteristics of MGD019 in cynomolgus monkeys were similar to those of humanized mAbs in this species, which is consistent with the possibility of dosing every 2 or 3 weeks in humans. More important, this animal model provided evidence of both PD-1 and CTLA-4 blockade, with a safety profile that compares favorably to published data of the combination of ipilimumab and nivolumab in this species.
Compared to animals treated with retifanlimab, the anti-PD-1 mAb precursor, animals treated with MGD019 demonstrated pharmacodynamic changes consistent with dual PD-1 plus CTLA-4 blockade, with no excess toxicity. These included an increased number of proliferating (Ki67+) circulating T cells, accompanied by increased spleen weights attributed to T cell expansion. MGD019-treated animals also show circulating T cells with phenotypes associated with CTLA-4 blockade, including the induction of ICOS+ CD4 cells and the expansion of the memory-like phenotype. Consistent with its design, the IgG4-bearing molecule showed no reduction in tissue T reg numbers. Our results indicate a range of immunostimulating properties of MGD019 independent of Treg depletion. Administration of up to 100 mg/kg was well tolerated, with minimal...
evidence of watery feces at the highest doses. By contrast, the combination of ipilimumab and nivolumab resulted in persistent diarrhea and gastrointestinal tract inflammation at doses as low as 3 mg/kg ipilimumab plus 10 mg/kg nivolumab, with the 10 mg/kg ipilimumab plus 50 mg/kg nivolumab dose exceeding the highest non-severely toxic dose.40

A relatively low first-in-human dose of 0.03 mg/kg was chosen due to the potential for unexpected toxicity of dual blockade. The experience in the dose escalation phase of the ongoing first-in-human evaluation of MGD019 has demonstrated acceptable safety of MGD019 at doses up to 10 mg/kg without protocol-defined dose-limiting toxicities (i.e., prespecified events occurring during the first 28 days after initial MGD019 administration). However, subsequent to additional enrollment at the top dose level (i.e., 10 mg/kg), intolerability was observed in patients with delayed grade 3 TRAEs, notably events of myocarditis and enterocolitis occurring in separate patients with onset at study days 74 and 81, respectively. Immune-related adverse events have been manageable, and patients have recovered without sequela after immunosuppressive treatment and MGD019 interruption or discontinuation, as appropriate.

Although the maturing MGD019 clinical safety data correspond to a small population treated to date at various dose levels (n = 33), the overall tolerability and incidence of grade ≥3 TRAEs compares favorably to published ipilimumab plus nivolumab safety data.10 Human PK of MGD019 was confirmed to be similar to that of recombinant mAbs, including a linear profile and a 12.4-day half-life. Full PD-1 blockade was achieved at doses ≥1 mg/kg every 3 weeks (Q3W). ICOS upregulation, a surrogate measure of CTLA-4 blockade (CTLA-4+ cells are undetectable in the circulation; data not shown), was induced by MGD019 at doses ≥3 mg/kg. The association between the CTLA-4 blockade biomarker (ICOS induction) and objective clinical responses suggests that CTLA-4 blockade may drive the clinical benefits of the bispecific in this patient population. The role of ICOS induction as a response biomarker warrants further investigation.

The frequency of severe TRAEs and overall safety profile of MGD019 is favorable for this class of agents. At doses <10 mg/kg, the safety profile has been generally consistent with that of anti-PD-1 monotherapy. At doses ≥3 mg/kg, MGD019 demonstrated evidence of anti-tumor activity in this pre-treated patient population, with responses observed in patients with low mutational burden colorectal cancer or those having failed prior checkpoint inhibitor therapy.

The encouraging preclinical and initial clinical data support further clinical investigation of the potential of MGD019, a purpose-designed single agent to inhibit both PD-1 and CTLA-4, to improve patient outcomes through increased tolerability and therapeutic responses.

Table 2. Treatment-Related Adverse Events in ≥5% of Patients and AESI in Order of Decreasing Frequency

<table>
<thead>
<tr>
<th>TRAE</th>
<th>3 mg/kg No. (%)</th>
<th>6 mg/kg No. (%)</th>
<th>10 mg/kg No. (%)</th>
<th>Total (All Dose Levels) No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All Grades (N = 7)</td>
<td>Grade 3* (N = 7)</td>
<td>All Grades (N = 3)</td>
<td>Grade 3* (N = 3)</td>
</tr>
<tr>
<td>Any adverse event</td>
<td>7 (100)</td>
<td>2 (28.6)</td>
<td>3 (100)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>2 (28.6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nausea</td>
<td>1 (14.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pruritus</td>
<td>1 (14.3)</td>
<td>0</td>
<td>1 (33.3)</td>
<td>0</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>2 (28.6)</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rash maculo-papular</td>
<td>2 (28.6)</td>
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IRR, infusion-related reaction.

*aNo grade 4 or grade 5 TRAEs observed as of the data cutoff.
*bAdverse event of special interest.
Limitations of Study

While the initial clinical data indicate that MGD019 is well tolerated and yields anti-tumor activity, additional patients will need to be evaluated to substantiate these preliminary findings and determine the consistency and durability of observed responses. The present study is also limited by the unavailability of on-treatment tumor biopsies. The evaluation of such biopsies will facilitate the dissection of the MGD019-mediated mechanism of action in the TME, pursuant to the hypothesis of MGD019 preferentially targeting dual PD-1/CTLA-4-expressing T cells in the context of CTLA-4 blockade. Future studies will also explore correlations of response with baseline biomarkers, including PD-1 and CTLA-4 co-expression on TILs and peripheral pharmacodynamic responses to PD-1 and CTLA-4 blockade, as well as attempt to define patient populations with potential improved therapeutic benefit to MGD019.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xcrm.2020.100163.

ACKNOWLEDGMENTS

The authors wish to thank Penelope Bristow for assistance with editing and formatting; Kerri Cali, Susan Brann, Joanna Lohr, and Pepi Pencheva for operational management of the Phase I study; Douglas Smith, Gurunad Chichilli, Qin Tang, Sharad Sharma, Alan Reduta, and Christine Shoemaker for their help in developing this project; and Syd Johnson (1957–2019) for inventing the DART platform. Research funding was provided by MacroGenics.

AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS


Received: September 6, 2020
Revised: October 28, 2020
Accepted: November 25, 2020
Published: December 22, 2020

REFERENCES


### Key Resources Table

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### Chemicals, Peptides, and Recombinant Proteins

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### Experimental Models: Cell Lines

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**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Paul A. Moore (moorep@macrogenics.com).

**Materials Availability**
Limited quantities of newly generated materials associated with the paper are available under MTA.

**Data and Code Availability**
This study did not generate/analyze datasets or code.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell Lines and Cell Culture**
Cell lines expressing human PD-1 (Jurkat/PD-1), human CTLA-4 (Jurkat/CTLA-4) and co-expressing human PD-1 together CTLA-4 (Jurkat/PD-1+CTLA-4) were provided by Promega (Madison, USA) as a part of bioassay systems and cultured according to manufacturer’s instructions. U20S cells engineered to express PD-1 and CTLA-4 were obtained from DiscoveRx (Fremont, USA) and cultured using media provided by the manufacturer.

**Human and Non-human Primates Tissue Samples**
Normal human tissue microarray and tumor microarrays of ovarian, breast, lung, colon, rectal cancer samples were provided by Advanced Cell Diagnostic (Newark, USA). Heparinized healthy donor blood was purchased from StemExpress (Folsom, USA). PBMCs were isolated by density centrifugation in Ficoll Paque (GE Healthcare, Chicago, USA). Cryopreserved dissociated tumor cells from patients with lung, renal, ovarian, and colorectal carcinomas were obtained from Discovery Life Sciences, Inc. (Los Osos, USA). The use of these materials does not require IRB approval. Sections of spleen were collected during necropsy from cynomolgus monkeys used in the GLP toxicology study. Spleen sections were transported overnight at ambient temperature in serum-free RPMI media. Pieces were mechanically separated using 70 μM cell strainers into single cell suspensions.

**Cynomolgus Monkeys**
The nonclinical toxicology study was conducted at Charles River Laboratories (CRL), Mattawan, USA, in accordance with US Food and Drug Administration Good Laboratory Practice Regulations for Nonclinical Laboratory Studies (21 CFR Part 58), the US Department of Agriculture Animal Welfare Act (9 CFR Parts 1, 2, and 3), and the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources. The study protocol was approved by the CRL Institutional Animal Care and Use Committee.

**MGD019 Phase 1 Study Participants**
The clinical study entitled A Phase 1, First-in-Human, Open-Label, Dose Escalation Study of MGD019, a Bispecific DART Protein Binding PD-1 and CTLA-4 in Patients with Unresectable or Metastatic Neoplasms was approved by IntegReview IRB and registered on https://www.clinicaltrials.gov (Identifier: NCT03761017). Male (n = 17) and female (n = 16) patients (median age 61 years) with histologically proven, unresectable, locally advanced or metastatic solid tumors for whom no approved therapy with demonstrated clinical benefit is available or patients who are intolerant to standard therapy. Measurable disease, Eastern Cooperative Oncology Group performance status 0-1, life expectancy > 12 weeks.
**METHOD DETAILS**

**Quantification of PD-1 and CTLA-4 mRNA expression**

RNAscope®2.5 HD duplex ISH tissue profiling of PD-1 and CTLA-4 in ovarian, breast, colorectal, lung cancer and normal tissues was performed by Advanced Cell Diagnostic, Inc. (Newark, USA) according to published protocol. FFPE tumor or normal tissue cores were mounted on slides that were pretreated with enzyme (protease) prior to hybridization with oligonucleotide probes targeted to the RNA in the sample. A series of wash steps followed, to amplify the signal. In the chromogenic assay, detection is via a chromogenic substrate, which produces a precipitate visible under common bright-field microscopy at 10-20X magnification, forming distinct red or green dots. Brightfield images were acquired using an AperioAT2 digital slide scanner equipped with a 40x objective. Software analysis was performed with HALO software (IndicaLabs, USA) to provide cell by cell quantitative results. Cells expressing two or more hybridization dots for each probe were considered positive.

**Flow Cytometry**

Cells were stained with Fixable Viability Dye-780 (Fisher Scientific, Waltham, USA) and FACS Abs (Key Resources Table). PFA-based fixation/permeabilization buffer system (Invitrogen, Carlsbad, USA) was used for intracellular staining, including FoxP3. Whole blood from patients treated with MGD019 was collected and stabilized using Cyto-CheX BCT tubes (Streck Corp., La Vista, USA). Staining was performed in whole blood followed by fixation and red blood cells lysis with Pharm Lyse buffer (BD Biosciences, San Jose, USA). Samples were analyzed using LSRFortessa cytometer (BD Biosciences, San Jose, USA); at least 20’000 events were collected for each sample.

**Receptor Occupancy Studies**

One hundred µL of whole blood samples (per time point/per patient) was incubated with saturating concentration of MGD019 or control DART, followed by lysis and detection of MGD019 by biotinylated anti-drug mAb (anti-EK coil) and Strep-PE in “MGD019-spiked” and control samples. After subtraction of background fluorescence (Strep-PE only), receptor occupancy (RO) values were calculated as a fraction of maximal binding capacity: \( RO = \frac{[\text{MFI(PE)} \text{ of untreated sample} – \text{background MFI(PE)}]}{[\text{MFI(PE)} \text{ of “MGD019-spiked” sample} – \text{background MFI(PE)}]} \).

**In vivo PD-1 Blockade Studies**

Patients whole blood samples (per time point/per patient) were incubated with commercial, APC-labeled, MGD019-competing anti-PD-1 mAb (clone J105, eBioscience, San Diego, USA). Percent of CD3*CD4* lymphocytes stained with commercial anti-PD-1 mAb before and after MGD019 administration was recorded for each patient.

**Molecular Design of MGD019**

MGD019 is a tetravalent Fc-bearing DART molecule comprising two PD-1 and two CTLA-4 binding domains. The PD-1 binding domain is derived from retifanlimab, which was generated by immunizing mice with His-tagged human PD-1 extracellular domain and standard hybridoma technology. The murine mAb was humanized by CDR grafting. The CTLA-4 binding domain is derived from human mAb 4B6. The Fc domain of MGD019 is of an IgG4 isotype with S228P mutation to prevent dissociation of the Fc dimer and M252Y/S254T/T256E mutations to enhance MGD019’s in vivo half-life. MGD019 was produced in ExpiCHO or CHO-K1 cells and purified by Protein A affinity chromatography, followed by size exclusion chromatography or ion exchange chromatography using standard procedures.

**Primary SEB Assay**

Cryopreserved healthy donor PBMC were thawed and plated 10⁵ cells/well in 200 µL of complete RPMI. mAbs and bispecific inhibitors were added at fixed concentration (10 µg/mL), and Staphylococcus Aureus Enterotoxin B (SEB, Toxin Technology, Inc., Sarasota, USA) was titrated as indicated. Cells were incubated for 96 hours prior to supernatant collection.

**SEB Restimulation Assay**

Freshly isolated PBMC were activated with 0.5 ng/mL SEB for 48 hours, followed by extensive washing and restimulation with 0.5 ng/mL SEB in the presence of test molecules. Supernatants were collected 48 hours after incubation.

**Mixed Lymphocyte Reaction**

CD14+ cells were isolated from human PMBC using positive selection kit (Milteniy Biotech, Bergisch Gladbach, Germany) and cultured for 7 days in vitro in the presence of GM-CSF and IL-4, 100 and 50 ng/mL respectively (PeproTech, Inc., Rocky Hill, USA). Seven days after plating cells were collected and seeded 2 × 10⁴ per well into 96 well plate. CD4+ T cells were freshly isolated negative selection kit (Milteniy Biotech, Bergisch Gladbach, Germany) from unrelated donor PBMC and co-plated with APCs in the presence of test molecules. Supernatants were collected at 96 hours after incubation.
Depletion of Autologous Treg cells
Freshly isolated PBMC were plated in complete RPMI at 10^6 cells per mL and stimulated with CD3 beads (Invitrogen, Carlsbad, USA) in the presence of indicated mAbs or MGD019 at 1 ug/mL. 48 hours later cells were collected and stained with CD4 and FoxP3 mAbs.

Cell Surface Binding and Ligand Blockade
Jurkat/CD3, Jurkat/CTLA-4 and Jurkat/CD3+CTLA-4 were generated by stable transfection of parental cells. Primary T cell were purified from PBMC using negative selection procedure and stimulated with CD3 beads (both Invitrogen, Carlsbad, USA) in complete RPMI supplemented with 300 IU/mL of IL-2 (PeproTech, Inc., Rocky Hill, USA) to induce expression of CD3 and CTLA-4. Cells were incubated with titrated mAbs or DART molecules and detected with secondary goat anti-human IgG polyclonal antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, USA). For competition studies cells were incubated with 1 ug/mL biotinylated re-combinant B7-1 or PD-L1 (BPS Bioscience, San Diego, USA) in the presence of unlabeled test molecules and detected with Streptavidin/R-PE. Flow cytometry was performed using FACSCanto II cytometer (BD Biosciences, San Jose, USA) in plate format; at least 20'000 events were collected for test well.

MGD019-mediated Ligand Blockade
PD-1, CTLA-4 and PD-1+CTLA-4 bioassay systems were obtained from Promega (Madison, USA) and used according to manufacturers’ instructions. A CHO-based stimulator line expressing anti-CD3 and checkpoint ligands (PD-L1, B7-1 or both) and a Jurkat-based reporter cell line were cultured together in the presence of MGD019 or mAbs. Induction of luciferase under control of NF-AT or IL-2 promoter was detected using Steady Glo substrate. U2OS PD-1/CTLA 4 Dimerization Assay. The PathHunter® dimerization assay (DiscoveRx, Fremont, USA) utilizes the enzyme fragment complementation technology where two split β-gal fragments, which independently had no enzymatic activity, could be formed back into a functional β gal to generate chemiluminescence. U2OS cells were engineered to stably coexpress fragments-tagged CTLA-4 and PD-1; the dimerization assay was performed in the presence of test articles at indicated concentrations according to manufacturer’s instructions.

Cynomolgus Monkey Toxicity Study
A 4-week, repeat-dose study was conducted in cynomolgus monkeys (Macaca fascicularis) to evaluate the toxicity of MGD019. Forty cynomolgus monkeys of Chinese origin were randomly assigned to 4 groups (5/sex/group) to achieve similar group mean body weights. The animals were dosed with the vehicle (5% dextrose injection) or MGD019 at 10, 40, or 100 mg/kg via IV infusion for 30 minutes once weekly for a total of 4 doses (days 1, 8, 15, and 22). After the completion of dosing, a subset of animals (2/sex/group) underwent a 10-week recovery period to evaluate the persistence or delayed occurrence of effects. In-life evaluations included clinical signs, body weights, food consumption, neurobehavioral, electrocardiographic and ophthalmic examinations, vital signs assessments, clinical chemistry, hematology, urinalysis, PK, ADA, and peripheral blood immunophenotyping. A full necropsy was conducted for all animals, with organs weighed and tissues collected, preserved, and processed for histopathologic evaluation. Samples of spleen were collected from each animal for splenocyte immunophenotyping.

MGD019 PK studies
Intact MGD019 serum concentrations were measured by bispecific enzyme-linked immunosorbent assay at indicated time points. Open one- or two-compartment IV infusion model was employed to fit the PK data using actual times and concentrations, actual infusion times, and nominal doses. Individual first dose data were modeled and weighted reciprocally of predicted concentration squared (–2). For PK simulations mean values of best estimates of the model parameters were used for potential clinical dose range of 3 to 10 mg/kg and Q3W infusions.

MGD019 Phase 1 Clinical Study
The clinical study entitled A Phase 1, First-in-Human, Open-Label, Dose Escalation Study of MGD019, a Bispecific DART Protein Binding PD-1 and CTLA-4 in Patients with Unresectable or Metastatic Neoplasms was approved by IntegReview IRB and registered on https://www.clinicaltrials.gov (Identifier: NCT03761017). Objectives. To characterize safety of MGD019 given IV to patients with advanced cancers, as well as PK and preliminary antitumor activity of MGD019 using conventional RECIST 1.1 and immune-related (ir)RECIST.

Enrollment criteria. Patients with histologically proven, unresectable, locally advanced or metastatic solid tumors for whom no approved therapy with demonstrated clinical benefit is available or patients who are intolerant to standard therapy. Measurable disease, Eastern Cooperative Oncology Group performance status 0-1, life expectancy > 12 weeks. Study design. MGD019 was evaluated in sequential, escalating, weight-based doses ranging from 0.03 mg/kg to 10 mg/kg in successive cohorts of 3 to 9 patients each in a 3+3 design. MGD019 is administered as an IV infusion over 30 minutes every 3 weeks during a 24-week Induction Period. Safety assessment is based on AEs from initiation of study drug through 30 days after last study drug or until start of subsequent anticancer therapy. Tumor assessments are obtained using CT and/or MRI scans (cutaneous lesions may be measured using callipers and/or photographs with an included scale). During the Induction Period, tumor assessments occur at 12 and 18 weeks after treatment initiation. After confirmation of the safety of the dose level, additional patients were added to the 3 and 10 mg/kg cohorts to gather additional safety, PK, and PD data.
QUANTIFICATION AND STATISTICAL ANALYSIS

The unpaired and nonparametric Mann-Whitney test with two tailed p value calculation was used to measure differences between two groups. For multiple group comparisons, one-way ANOVA or two-way ANOVA was used to determine statistically significant differences between samples. Measurements were summarized as mean ± SD as noted in figure legends. Experimental sample numbers (n) are indicated in the figure legends. Additional or alternative statistical analysis methods are described in the individual figure legends. Graph generation and statistical analysis were performed using GraphPad Prism software (GraphPad, La Jolla, USA).

ADDITIONAL RESOURCES

The First-in-Human clinical trial was registered on www.clinicaltrials.gov (Identifier: NCT03761017) https://clinicaltrials.gov/ct2/show/NCT03761017