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Article

Preclinical evaluation of MORAb-003, a humanized monoclonal antibody antagonizing folate receptor-alpha

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The highly restricted distribution of human folate receptor-alpha (FRα) in normal tissues and its high expression in some tumors, along with its putative role in tumor cell transformation, make this antigen a suitable target for antigen-specific, monoclonal antibody-based immunotherapy for oncology indications. We have developed a therapeutic humanized monoclonal antibody with high affinity for FRα, named MORAb-003, which was derived from the optimization of the LK26 antibody using a whole cell genetic evolution platform. Here we show that MORAb-003 possesses novel, growth-inhibitory functions on cells overexpressing FRα. In addition, MORAb-003 elicited robust antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) *in vitro*, and inhibited growth of human ovarian tumor xenografts in nude mice. Because of its multimodal activity *in vitro* and its safe toxicology profile in non-human primates, MORAb-003 development has recently been advanced to clinical trials involving ovarian cancer patients.

Keywords: preclinical drug evaluation, monoclonal antibody, folate receptor, antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity

Introduction

Ovarian cancer is the leading cause of death among women with gynecologic malignancies and, according to the American Cancer Society, it will account for more than 15,000 deaths this year alone. Because its initial presentation is often at an advanced stage and despite advances in chemotherapy, the majority of subjects eventually die of the disease. The low rate of long-term survival (15% to 30%) is due to the frequent recurrence of the disease in the abdominal cavity, as well as to the intrathoracic metastases. The median survival of patients with optimally debulked ovarian cancer has recently been measured at 57.4 months (1). New therapeutic agents that are effective and well tolerated are clearly needed. Cancer antigen-specific monoclonal antibodies that have direct pharmacologic effects or can stimulate immunological responses represent a promising class of agents for acute therapy or chronic maintenance of remission.

The human folate receptor-alpha (FRα) is overexpressed in ovarian cancer but largely absent in normal tissue (2-8). It is hypothesized that the presence of elevated levels of FRα can be correlated with the propagation rate and phenotype of the tumors. Corroborating this hypothesis are the following evidence: (i) the degree of FRα expression is associated with the parameters of biological aggressiveness of ovarian cancer (8); (ii) anti-FRα single-chain antibody, intracellularly expressed, was able to reverse the transformed phenotype of ovarian cancer cells (9); (iii) under certain conditions, cells may depend on the FRα to obtain enough 5-methyltetrahydrofolate for growth (10); (iv) there is a correlation between human ovarian carcinoma growth and FRα overexpression (11); and (v) in ovarian cancer cell lines, a statistically significant inverse relationship exists between FRα overexpression and cisplatin resistance (12).

A number of monoclonal anti-human FRα monoclonal antibodies have been reported in the literature. Previous clinical studies of a FRα binding antibody, MOv18 (either murine or chimeric), administered to women with ovarian cancer, have been reported (13-20). The results from these studies have suggested that an unconjugated or radiolabeled FRα binding antibody does not produce overt toxicity and can provide clinical benefits to this group of patients.

Immunohistochemistry studies using LK26, a distinct murine antibody to the human FRα, demonstrated that the expression of this receptor in normal tissues was restricted to the trophoblast, while FRα was found expressed on all cultured choriocarcinoma, teratocarcinoma and renal cancer cell lines (6). Moreover, it was determined that this antigen was not expressed in most cell lines derived from other tumor types or in cultured normal kidney epithelium cells and fibroblasts (6). Among cancers, FRα was found to be expressed in ovarian and renal cancers, and in choriocarcinoma and teratocarcinoma tissues (6). The highly restricted distribution of FRα in normal tissues and its expression in tumors suggested that this antigen may have potential as a diagnostic marker of gestational choriocarcinoma and germ-cell tumors and, more importantly, as a target for passive immunotherapy. Clinical investigation of LK26 was later abandoned because its initial humanized version showed a drastically reduced affinity of 0.2 μM, therefore hampering any future development.

MORAb-003 is an optimized anti-FRα monoclonal antibody generated by applying the *morphogenics* technology (21) to the original cell line producing the suboptimal humanized LK26 antibody. After the optimization process, MORAb-003 exhibited an affinity similar to the original murine LK26 antibody (approx. 2 nM) and a tissue binding profile consistent with the distribution of the folate receptor (3, 6, 8, 22, 23). In this

report, we describe the *in vitro* and *in vivo* activity exerted by MORAb-003 and show that its binding to the folate receptor causes a reduction in the cellular growth rate, elicits antibody dependent cellular cytotoxicity (ADCC) and complement mediated cytotoxicity (CDC), reduces tumor growth of human tumor xenografts in nude mice, and causes no observable toxicity in cynomolgus monkeys. Because of its promising preclinical profile, MORAb-003 is now being evaluated in the clinic as a potential therapeutic agent for ovarian cancer.

Results

Generation of MORAb-003

The initial humanized form of LK26 antibody (IgG1κ) was expressed in NS0 cells and exhibited a reduced steady-state dissociation constant (K_D) of 0.2 μM (Figure 1A). The cell line producing this antibody was obtained from the Ludwig Institute for Cancer Research (New York, NY) and used to generate new expression cell lines in the pursuit of improving antibody activity and productivity. To this aim, cells were transfected with a DNA vector for the expression of the dominant negative, truncated form of the human *PMS2* gene, named morphogene, to inhibit the DNA mismatch repair process and enhance the mutational rate as previously described (21, 24). Upon ELISA screening to identify cell clones secreting antibodies with enhanced binding to the FRα antigen, one clone, among others, was selected for further characterization. This clone was found to secrete an antibody showing an increased K_D of 2.2 nM (see Figure 1B for binding kinetics), which was very similar to the affinity of the original murine LK26 antibody (Figure 1A).

The antibody secreted by this line was named MORAb-003 and, surprisingly, on the primary nucleotide and amino acid level it did not differ from the original humanized LK26. However, analyses of RT-PCR-amplified MORAb-003 transcripts showed the absence of an alternative light chain splice form that was instead found in the transcripts of the parental humanized LK26 antibody (data not shown). It is possible that a mutation caused by inhibition of the DNA repair process induced by the morphogene silenced the expression of this alternate transcript. We speculated that this fairly abundant splicing variant caused perturbation of the parental antibody tetramer assembly, resulting in a mixture of active and inactive molecules which lowered the antibody affinity as a net result. This discovery enabled us to focus on the optimization of antibody expression vectors and cell line expression systems, resulting in the generation of CHO cells producing a high affinity MORAb-003 antibody.

MORAb-003 inhibits FRα-dependent cell growth

It has been reported that overexpression of human FRα in murine NIH/3T3 cells enhances their growth properties both *in vitro* and *in vivo* (11). We explored the possibility that this FRα activity could also be seen in hamster cells and could be modulated by MORAb-003 binding to FRα. For this purpose, we transfected CHO cells with DNA vectors for the expression of the wild type membrane-bound form of FRα. Figure 2A shows FACS analyses of CHO cells expressing FRα (CHO-FR) confirming high expression of the receptor on the cell surface, while no expression was found in parental CHO cells (data not shown). Next, we cultured CHO-FR and parental CHO cells for 72 hours in low folate (<20 nM) medium to decrease the intracellular folate pool. Cells were then fed increasing concentrations of the folic acid metabolite 5-methyltetrahydrofolate (5-MTF) and cultured a further 96 hours. Under these conditions CHO-FR cells exhibited a reduced requirement for folate as demonstrated by an EC50 of 6.23 nM versus 339 nM for the parental CHO cells (Figure 2B). This 54-fold lower EC50 was consistent with the ability of CHO-FR cells to grow even when the concentration of folate was dropped from 1 μM to 10 nM, which corresponds to physiologic folate levels, whereas growth of parental CHO cells was halted at this lower concentration (Figure 2C). Addition of MORAb-003 to the CHO-FR culture increased folate EC50 in a dose-dependent fashion, suggesting that the binding of MORAb-003 to human FRα modulates its cell growth functions (see Discussion) while such an increase in folate EC50 was not observed in parental CHO cells (Figure 2D).

MORAb-003 mediates tumor cytotoxicity via CDC and ADCC *in vitro*

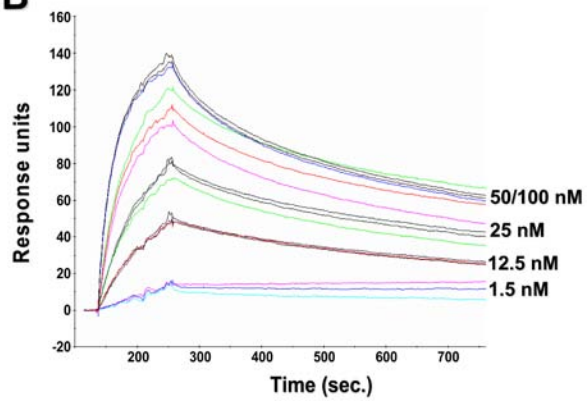
Important mechanisms of *in vivo* tumor cell cytotoxicity are believed to be mediated through the antibody-dependent fixation of complement factors and their induction of cell lysis (CDC), and through antibody opsonization of tumor cells followed by the recruitment of immune killer cells which can elicit cell-mediated cytotoxicity (ADCC). To assess the ability of MORAb-003 to elicit these two effector functions, we used human cancer cell lines as targets for CDC and ADCC studies. FRα was expressed in all ovarian cancer lines we tested, albeit at different levels, whereas no expression was observed in a human breast cancer line, ZR75-1 (Figure 3A). To measure CDC

Figure 1

A

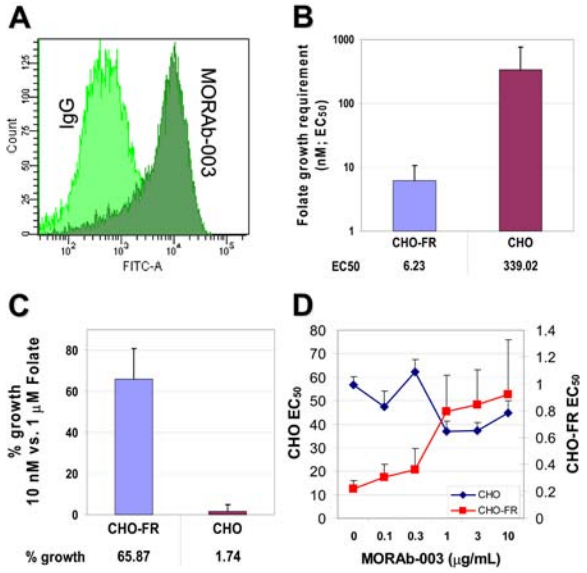
mAb	k_a (1/Ms)	k_d (1/s)	K_A (1/M)	K_D (M)
murine LK26	4.65E+05	3.25E-03	1.43E+08	7.00E-09
humanized LK26	8.19E+04	1.70E-02	4.82E+06	2.08E-07
MORAb-003	2.25E+05	5.02E-04	4.48E+08	2.23E-09

B



Determination of apparent steady-state dissociation constants for the interaction of anti-FRα antibodies with human folate receptor-alpha. (A) Rate constants and steady-state dissociation constants measured using immobilized, native FRα, and the indicated antibodies as analyte. For each experiment, 5 concentrations of antibody were assayed in triplicate. (B) Sensorgram from analysis of MORAb-003 binding kinetics to immobilized, native FRα. MORAb-003 concentrations are indicated next to the curves.

Figure 2



MORAb-003 inhibits FRα-dependent cell growth. (A) FACS analyses of CHO-FR cells stained with MORAb-003 or isotype control IgG confirming high expression of the receptor on the cell surface. (B) CHO-FR and parental CHO cells were cultured for 72 hours in low folate (<20 nM) medium and then fed increasing concentrations of 5-MTF. After 96 hours, EC50 was calculated as an average of three experiments. (C) CHO-FR and parental CHO cells were grown in the presence of 10 nM or 1 μM of 5-MTF. Growth at 10 nM 5-MTF is presented as a percentage of growth measured at 1 μM 5-MTF. (D) CHO-FR and parental CHO cells were cultured as in (B) and EC50 were measured in the presence of increasing concentrations of MORAb-003. MORAb-003 treatment (blue line) did not significantly change 5-MTF EC50 in parental CHO cells, while it increased 5-MTF EC50 in a dose-dependent fashion in CHO cells expressing human FRα (red line).

activity, IGROV-1 cells were treated with 10 μg/ml of MORAb-003 in the presence of 20% of fresh human serum and incubated for 1 hour at 37°C. Human serum and MORAb-003 concentrations were selected after dose titration to find optimal killing. The combination of MORAb-003 and human complement resulted in significant killing (46%) of IGROV-1 cells, as compared to either complement or MORAb-003 treatment alone (Figure 3B). Since IGROV-1 cells highly express complement-inhibitory proteins, including CD54 (data not shown), which can partially inhibit complement fixation, we measured MORAb-003-mediated CDC activity in non-tumor cells by employing CHO-FR as target cells. Under similar conditions, MORAb-003 activity resulted in a 98% cytotoxic effect on CHO-FR cells (Figure 3C) compared to 46% observed in IGROV-1 cells (Figure 3B), unveiling a greater potential of MORAb-003 to mediate CDC activity, depending on the cellular context.

To determine ADCC activity, target cells were labeled with ^{51}Cr and treated with MORAb-003 or isotype control IgG. Human PBMCs (effector cells) from normal donors were added at different target to effector cell ratios. Significant MORAb-003-specific cytotoxicity was observed against SKOV-3 and OVCAR-3 cells (Figure 4A), with maximal activity seen at a 1:50 target:effector ratio. MORAb-003 also mediated robust ADCC activity (>70% cytotoxicity) on IGROV-1 cells, but had no effect on the FRα-negative breast cancer cells, ZR75-1 (data not shown). No significant cytotoxicity was detected with a control IgG. Titration of the antibody showed that MORAb-003-specific ADCC activity could be seen at a concentration as low as 0.1 μg/ml (Figure 4B). Overall, these data suggest that MORAb-003 can elicit specific CDC, as well as ADCC, activity *in vitro*.

Murine LK26 antibody reduces growth of SKOV-3 xenografts in nude mice

To explore the potential anti-tumor effect of targeting FRα *in vivo*, and assuming that effector functions in mouse are better mediated by a murine antibody rather than by its humanized version, we employed the murine LK26 antibody (precursor of MORAb-003) to treat nude mice implanted with SKOV-3 cells. Animals were treated with vehicle, isotype-matched normal murine IgG or LK26 following a regimen consisting of 0.1 mg/dose administered three times a week for 4 weeks, while monitoring for tumor volumes twice a week for up to 2 months. As shown in Figure 5, a significant reduction (approx. 62%) of tumor growth was observed with the LK26 treatment (0.1 mg/dose) compared to an equivalent dose of the normal IgG control ($P < 0.05$). Higher doses of LK26 led to an overall increase in tumor suppression; however this effect could not be unequivocally interpreted because comparable high doses (50 mg/kg) of the control IgG showed some unexpected non-specific anti-tumor effect (data not shown).

Non-human primate toxicology

Immunohistochemistry studies determined that MORAb-003 infrequently stained the tubular epithelium, epithelium of the fallopian tube, and duct epithelium of the pancreas in both normal human and cynomolgus monkey tissues. Cynomolgus monkey was therefore considered an appropriate toxicology model. After the intravenous infusion (one hour, twice per week for 4 weeks) of either 2.86, 8.55 or 17.1 mg/kg of MORAb-003, all physical observations, clinical chemistries, body weights, food consumption, gross pathology and histopathologies were considered to be normal. The only exception was a dose-related increase in spleen size and in the number of lymphoid follicles in the white pulp. Hyperplasia of the lymphoid zone of the spleen is a known effect of injection of antigenic or immune stimulant (25) and considered to be due to foreign proteins. Other lymphoid tissues, including the mesenteric and mediastinal lymph nodes and the thymus, did not show any apparent MORAb-003-related alterations in lymphocyte populations. The absolute and relative kidney weights were higher, but there were no changes in blood urea nitrogen and creatinine levels. The splenic changes observed in the animals sacrificed immediately post-infusion were not seen in the 28-day recovery animals. The size and number of splenic lymphoid follicles were similar in control and MORAb-003-treated animals, indicating recovery from any lymphocyte stimulation caused by the antibody. In fact, the number and size of lymphoid follicles in the control recovery animals was similar to that seen in most of the test antibody-treated animals at the end of dosing, suggesting that the lymphoid stimulation seen at the end of dosing was within the normal physiological range. As no adverse events were observed, the no observed adverse effect level (NOAEL) was estimated to be greater than 136.8 mg/kg, over 28 days. The findings of a 24-week study were similar. No drug-related clinical, laboratory or pathologic changes were observed.

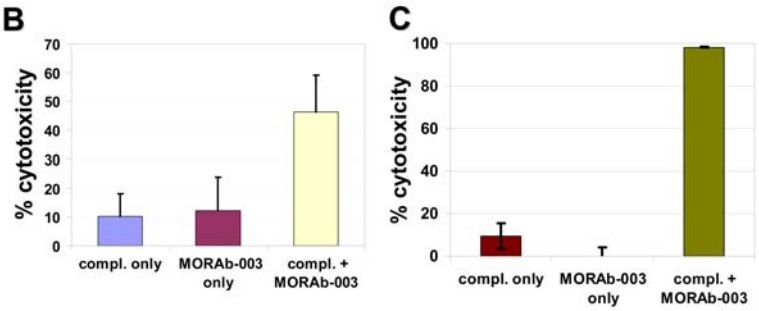
Toxicokinetics

The preclinical pharmacokinetic data are derived from the repeated dose toxicology studies in female cynomolgus

Figure 3

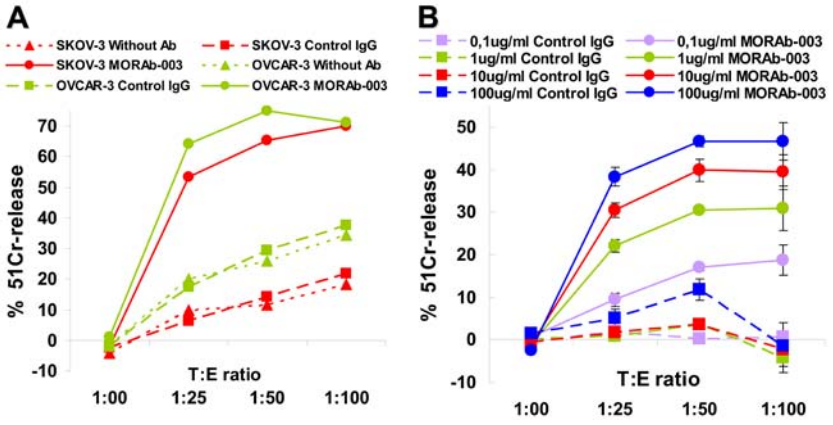
A

FACS Analyses		Median fluorescence	
Cell line	Description	Normal IgG	MORAb-003
OVCAR-3	Ovarian cancer	53	151
SKOV-3	Ovarian cancer	183	487
IGROV-1	Ovarian cancer	117	5882
ZR75-1	Breast cancer	219	244



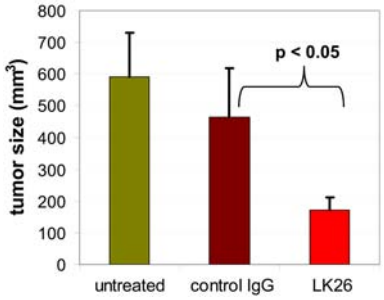
MORAb-003 mediates tumor cytotoxicity via CDC *in vitro*. (A) FACS analyses on ovarian cancer lines and one breast cancer line measuring FRα expression levels. Cells were stained with MORAb-003 or an isotype control IgG. (B) IGROV-1 cells were treated with 10 µg/ml of MORAb-003 in the presence of 20% of fresh human serum and incubated for 1 hour at 37°C. Cells were counted via trypan blue exclusion and the number of dead cells after treatment was expressed as the percentage of the number of viable cells before the treatment (% cytotoxicity). (C) MORAb-003 killed CHO-FR cells via a CDC mechanism (assay conditions as in panel B). Compl. corresponds to fresh human serum.

Figure 4



MORAb-003 mediates tumor cytotoxicity via ADCC *in vitro*. Target cells were labeled with ^{51}Cr and treated with MORAb-003 or an isotype control IgG. Effector cells were tested at different target to effector cells ratios (T:E ratio). (A) SKOV-3 or OVCAR-3 cells were used as target cells and treated with 10 µg/ml MORAb-003. (B) IGROV-1 cells were used as target cells and treated with concentrations of MORAb-003 ranging from 0.1 to 10 µg/ml.

Figure 5



LK26 antibody reduces growth of SKOV-3 cell xenografts in nude mice. SKOV-3 tumor cells (5 x 10^6) were injected subcutaneously into nude mice (10 animals per group). Mice were treated with vehicle, 0.1 mg/dose of isotype-matched normal murine IgG, or 0.1 mg/dose of LK26 (precursor of MORAb-003). On day 64, tumor measurements were converted to tumor volume (in mm^3) using the formula (W^2 x L)/2 . The *P* value was calculated using a double-tailed *t*-test.

Table 1

Mean pharmacokinetic parameters for MORAb-003.

MORAb-003 Dose (mg/kg/occasion)	MORAb-003 Total Dose (mg/kg)	$AUC_{0\text{-}578h}$ (µg.h/ml)	Cmax (µg/ml)
2.86	22.88	56600 (5060)	200 (28.0)
8.55	68.40	172000 (42300)	589 (176)
17.10	136.80	398000 (36000)	1280 (109)

monkeys. Pharmacokinetic parameters were assessed for MORAb-003 at doses of 2.86, 8.55 or 17.1 mg/kg twice weekly for four weeks. The means of maximum serum concentrations (Cmax) and the areas under serum MORAb-003 concentration-time curves were estimated for the period of 0 to 578 hours after the first dose. The areas under the curve ($AUC_{0\text{-}578h}$), with standard deviations (SD) in parentheses, are summarized in Table 1. Following intravenous infusions of MORAb-003 to female monkeys, the $AUC_{0\text{-}578h}$ and Cmax values increased proportionately to the dose increase. Over the approximately 3-

and 6-fold dose ranges, the $AUC_{0\text{-}578h}$ values for MORAb-003 increased 3.04- and 7.03-fold and Cmax values increased 2.95- and 6.40-fold. Two animals were followed for an additional 28 days after the last infusion. MORAb-003 was measurable in serum up to 4 weeks after the last infusion. The half-life of MORAb-003 was relatively long (239 and 289 hours as determined from the two recovery animals).

The animals were tested for the development of monkey anti-human antibodies in response to the administration of MORAb-003. In nine out of a total of eleven animals, there was no evidence of an anti-human antibody response following twice-weekly dosing of MORAb-003 for four weeks, up to a maximum dose of 17.1 mg/kg/dose. In two animals from the highest dose group of 17.1 mg/kg, low and transient anti-human antibody IgM responses could be detected that did not affect the observed MORAb-003 concentrations in serum. In addition, there was no evidence of a secondary IgG response in these animals.

Discussion

Novel therapeutic agents that are safe and effective are needed for the treatment of ovarian cancer, a leading cause of cancer death among women. Over the years, monoclonal antibodies have proven to be well-tolerated, disease antigen-specific, and able to deliver clinical benefits to patients with conditions that include chronic inflammation, lymphomas, and metastatic solid tumors (26). Here we report the preclinical evaluation of MORAb-003, a humanized monoclonal antibody antagonizing FRα to determine its suitability as an anti-tumor agent. MORAb-003 was able to mediate different biological responses *in vitro*, including inhibition of FRα-dependent cell growth, CDC, and ADCC activity, as well as reduce tumor growth in mice implanted with human ovarian cancer cells, while exhibiting no adverse effects in non-human primates.

Presently, the mechanism by which MORAb-003 increases the folate requirement of CHO-FR cells and affects their growth properties in low folate conditions is unknown. One hypothesis is that binding of MORAb-003 leads to perturbation of the signaling emanating from the FRα directed to intracellular factors involved in the regulation of cell proliferation. Miotti *et al.* have postulated that the GPI-anchored FRα abounds in lipid rafts and is associated with lyn kinase, as well as the α and β subunits of G protein (27). Intriguingly, the authors found that the phosphorylated (activated) form of lyn kinase that is associated with FRα was translocated out of the lipid rafts when FRα was targeted with either MOv19 or murine LK26 (precursor of MORAb-003), or when the rate of cell division had been reduced by overconfluence. This evidence suggests a role of FRα in intracellular signaling associated with cell proliferation. We have observed a similar effect on the redistribution of activated lyn kinase after MORAb-003 was bound to FRα on IGROV-1 cells (data not shown). In view of these facts and on the basis that FRα plays a secondary role in the total cellular folate intake, while a greater proportion of folate internalizes through reduced folate carrier proteins in cancer cells (28), one scenario can be postulated whereby at physiologic concentration of folate, cancer cells overexpressing FRα can gain a growth advantage over FRα-null cells (see Figure 1C, CHO-FR vs. parental CHO) not via an increased folate intake but rather through a FRα-mediated proliferation stimulus mediated by lyn kinase signaling. The potential importance of lyn kinase signal transduction in the transformation of prostate cancer has recently been reported (29). The fact that MOv19 also reduced folate intake in cells treated with this antibody (27) suggests that the receptor undergoes a functional change upon antibody binding that affects both signaling and folate intake. However, this does not indicate whether the stimulus for the intracellular signaling is delivered by folate itself or another unidentified ligand. Of note, MORAb-003 did not alter the *in vitro* cytotoxic effect of methotrexate in drug-sensitive ovarian cancer cells (data not shown), indicating that this antibody does not affect the intake of folate analogues through the FRα and could be used in combination with these drugs. Overall, our data and that of others clearly pinpoint a role of FRα in promoting cell growth *in vitro* under physiologic levels of folate and that this function could be antagonized by antibody binding to this receptor.

We have shown that the murine LK26 antibody was capable of reducing tumor growth in a xenograft model employing SKOV-3 human ovarian cancer cells (Figure 3). This model did not allow us to address the possible mechanism by which this anti-tumor effect is mediated, or whether higher doses of MORAb-003 could result in tumor shrinkage. Further studies are necessary to address these questions, as well as to test possible additive or synergistic anti-tumor activity of MORAb-003 in combination with chemotherapeutic agents.

Since "naked" antibodies do not carry payloads such as toxins or cytotoxic compounds, it is important that they exert anti-tumor activities through a variety of mechanisms. We have shown that MORAb-003 mediates CDC activity on tumor cells, albeit not with the same efficiency as when compared to targeting non-tumor cells. However, tumor cells could also be effectively killed by MORAb-003 via an ADCC mode of action. Considering also its inhibitory effect on FRα-dependent growth, MORAb-003 *in vitro* activity fits the multimodal profile a naked monoclonal antibody requires to potentially exert pharmacological activity against tumor cells.

As part of a safety study it was necessary to determine the dose level at which potential toxicity in humans may occur due to binding of MORAb-003. In order to detect possible cross-reactivity, MORAb-003 was applied to a human tissue panel that included all the tissues recommended by the US FDA. The binding profile was essentially similar to that reported for other FRα-specific monoclonal antibodies, showing a very limited reactivity to normal tissues (data not shown). Since MORAb-003 does not bind to rodent tissues and its staining pattern was similar in human and cynomolgus monkey tissues, only non-human primates were used to determine potential toxicity. The results of these studies indicated that: (i) the estimated no observed adverse effect level for female cynomolgus monkeys was greater than 136.8 mg/kg over 28 days; (ii) no monkey anti-human antibody IgG responses were observed; (iii) toxicokinetic analysis indicated that serum levels were dose linear; and (iv) serum clearance was consistent with reported values for a humanized antibody in a cynomolgus monkey. This lack of toxicity can be explained by the fact that normal tissues, unlike tumors, do not overexpress FRα.

In view of the encouraging preclinical data obtained from our studies, we have initiated a Phase I clinical study to evaluate safety of MORAb-003, and more recently started a Phase II efficacy trial in chemotherapy-sensitive ovarian cancer patients using doses confirmed to be safe during Phase I.

Abbreviations

FRα, folate receptor-alpha; 5-MTF, 5-methyltetrahydrofolate

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Materials and methods

Purification of FRα

KB human nasopharyngeal carcinoma cells (ATCC # CCL-17) grown in T-300 flasks (Nalgene Nunc) were pelleted by centrifugation at 4°C in an Avanti J-20 XPI centrifuge, equipped with a SW20 (Sorvall) rotor, at 2,500 x *g* for 10 minutes. Extracellular liquid was discarded and the pellet was washed twice with 20 ml of ice-cold PBS, followed by centrifugation as above. Cells were then resuspended in hypotonic lysis buffer (10 mM potassium phosphate, pH 7.4) at 2 x 10^7/ml and incubated on ice for 15 minutes. Cells were disrupted by 10 passages through a syringe equipped with a 25G needle, and incubated on ice for 15 minutes. The lysate was centrifuged at 40,000 x *g* for 30 minutes at 4°C. The supernatant was discarded, and the pellet was washed once with 5 ml/10^7 cells of hypotonic lysis buffer, centrifuged as above, and resuspended in the same volume of hypotonic lysis buffer. The pellet was dissolved by addition of an equal volume of membrane solubilization buffer (10 mM potassium phosphate, 20 mM CHAPS, pH 7.4), and mixed by tumbling for 3 hours at 4°C. Insoluble material was removed by centrifugation at 40,000 x *g* at 4°C for one hour. The pH of the supernatant was lowered to 3.8 by addition of 0.5 M citric acid. The acidified supernatant was mixed with 10 mg/ml Norit-A charcoal, and stirred for one hour at 4°C, to remove endogenous bound folates. The charcoal was removed by centrifugation at 30,000 x *g* for 15 minutes and the clarified supernatant was adjusted to pH 7.4 by titration with 1 N NaOH. Proteins in the supernatant were buffer-exchanged into folate affinity binding buffer (10 mM potassium phosphate, 10 mM CHAPS, pH 7.4), using a 10DG desalting column (BioRad cat. 732-2010) equilibrated in folate affinity binding buffer. Folate affinity resin was prepared as previously described (30). Resin was packed into a Poly-Pac column (BioRad) and equilibrated with 10 volumes of folate affinity binding buffer, before applying the load at a rate of 0.5-1 ml/min. The column was washed sequentially with wash buffer 1 (10 mM potassium phosphate, 1 M NaCl, pH 7.4), wash buffer 2 (10 mM potassium phosphate, pH 7.4), and wash buffer 3 (1 mM potassium phosphate, pH 7.4) until A280 returned to baseline each time. Bound proteins were eluted by addition of 5 column volumes of elution buffer (10 mM acetic acid, 1 M NaCl). Fractions (0.5 ml/each) were collected in tubes containing 20 µl of neutralization buffer (1 M dibasic potassium phosphate). MORAb-003-reactive fractions were pooled, dialyzed for 6-18 hours against two changes of 100 volumes PBS at 4°C, and stored in aliquots at -80°C.

Surface plasmon resonance

The purified FRα was diluted into 10 volumes of 10 mM sodium acetate pH 4.0, and coupled to the surface of a research-grade CM5 chip (Biacore, Inc.) by standard amine chemistry (NHS-EDC, Biacore, Inc.), to a level of 50-250 response unit (RU) of antigen bound. The remaining active sites were quenched using 1 M ethanolamine. A reference flow cell consisting of an activated and quenched surface in the absence of antigen, or in the presence of BSA at a matched concentration to that in the previous step, was used to normalize readings. Purified anti-FRα antibodies were dialyzed overnight and diluted into HBS-EP buffer (Biacore, Inc.) to final concentrations of 1-100 nM. The running buffer used was HBS-EP. The on- (k_a) and off-rates (k_d) of each antibody were determined by observing the signal over time for triplicate injections of antibody at the concentrations indicated in Figure 1. Blank injections of HBS-EP were also performed to assess noise. All samples were randomly injected using the KINJECT command, and the dissociation phase of binding was observed for a minimum of 10 minutes. Since no mass transfer effects were obvious in control experiments and the use of more complex models failed to achieve statistically better fits, binding kinetics were extrapolated from a 1:1 Langmuir isotherm binding model using BiaEvaluation 4.1 software (Biacore, Inc.). Steady-state dissociation constants (K_D) were derived from the observed on- and off-rates using the BiaEvaluation software.

ADCC ^{51}Cr release assay

ADCC assays were conducted in the laboratory of Dr. Silvia vonMensdorff at Vrije University, The Netherlands. Donor peripheral blood mononuclear cells (PBMCs) used as effector cells were thawed and kept overnight in medium (IMDM supplemented with 10% fetal calf serum). The cells were resuspended in medium at a concentration of 10^7 cells/ml. The tumor cells used as target cells were detached from the culture flask and 10^6 cells in 100 µl fetal calf serum were labeled with 100 µCi (3.7 MBq) ^{51}Cr for 2 hours at 37°C. The cells were washed three times with 5 ml of medium and resuspended in medium at a concentration of 10^5 cells/ml. Fifty µl of the tumor cells were seeded in V-bottom 96-well culture plates. The cells were then incubated with 50 µl medium containing MORAb-003 or control antibody at concentrations indicated in the text. After 30 minutes of incubation at 37°C, 50 µl of the PBMCs were seeded in V-bottom 96-well culture plates at various target-effector cell ratios (1:0, 1:25, 1:50 and 1:100) and the plates were further incubated for 18 hours at 37°C. The release of ^{51}Cr in the

supernatant was determined in a gamma-counter. Each measurement was carried out in triplicate. Before ADCC testing, flow cytometric analysis of the cell lines with MORAb-003 was performed to establish antigen expression.

CDC assay

IGROV-1 cells (kindly provided by J. Bernard, Institute G. Roussy, Villejuif, France) were harvested using enzyme-free cell dissociation buffer (Invitrogen) and resuspended in RPMI 1640 medium with 2 mM L-glutamine and 10% heat inactivated FBS. The cells were counted, collected by centrifugation, and resuspended at a concentration of 5 x 10^6/ml in RPMI 1640. Seventy µl of cell suspension was combined with 20 µl of normal human serum (Innovative Research, MI) and 10 µl of MORAb-003 followed by incubation at 37°C for 2 hours. For negative controls, normal human serum was replaced with an equal volume of RPMI 1640 and MORAb-003 with an equal volume of PBS. Following incubation, the reaction mixture was diluted with 900 µl of PBS and the number of viable cells for each reaction was determined using a CEDEX automated cell counter (Innovatis, PA). Percent killing was calculated as follows: % cell killing = 100 x [1-(number viable cells treated/number viable cell untreated)]. All assays were carried out in quadruplicate.

Expression of FRα in CHO cells and folate-dependent growth inhibition studies

CHO cells were engineered to express surface FRα by transfection with an expression vector encoding for full length FRα. To generate stable transfectants, CHO-K1 cells were plated at 200,000 cells/well in a 6 well plate in 2 ml of complete RPMI (RPMI 1640 with 10% FBS and 2 mM L-glutamine) and incubated overnight at 37°C in 5% CO_2. The next day, transfections were carried out using Fugene 6 transfection reagent (Roche) according to the manufacturer's instructions. The following day, the medium was changed to selection medium (complete RPMI with 0.2 mg/ml Zeocin) and after one week of continuous selection the pool of cells was assayed for the expression of FRα by staining with FITC labeled MORAb-003, followed by FACS analysis. Single cell clones expressing FRα were generated by cell sorting into 96 well plates using a BD FACSaria. Clones were expanded, reanalyzed for FRα expression and a high-expression clone was chosen for further experiments.

Mouse xenografts studies

Human-mouse xenograft studies were conducted at the Institute of Drug Development (S. Antonio, TX). Female nude mice (nu/nu) between 5 and 6 weeks of age weighing approximately 20 g were obtained from Harlan, Inc. (Madison, WI). The SKOV-3 human ovarian carcinoma cell line was obtained from the American Type Culture Collection (ATCC) and derived from the cultivation of cells from the ascitic fluid of a 64 yr old. On day 0, animals were injected subcutaneously (s.c.) with 100 µl of a cell suspension containing 5 x 10^6 cells harvested from *in vitro* culture. On day 1, mice were randomized into their respective groups and treated with the various antibodies. All of the groups contained 10 mice and the animals were ear-tagged and followed individually throughout the experiment. LK26 antibody was administered intravenously (i.v) three times a week for 4 weeks at the doses indicated in the text. As a negative control, normal murine IgG was administered i.v. three times a week for 4 weeks. As an additional negative control, phosphate buffered saline (PBS) was given at a volume of 200 µl/mouse on the same schedule as the treatment groups. Mice were weighed twice weekly, starting on day 1. Beginning on day 5, tumor measurements were taken twice weekly. Tumor measurements were converted to mm^3 tumor volume by the standard formula, (W^2 x L)/2, where L and W are the major and minor diameters of the tumor expressed in millimeters, respectively.

Primate toxicity study

Female cynomolgus monkeys were studied as the first clinical indication explored is ovarian cancer. After a preliminary dose ranging study, a 28-day intravenous infusion (twice per week) toxicity study with a 28-day recovery period was completed in cynomolgus monkeys. In monkeys, the high intensity dosing (twice per week for 4 weeks) was designed to achieve high dosing levels over a short period of time to avoid the possible effects of the generation of monkey anti-human antibody response. The doses chosen represent 2 to 12 times the dose a human subject will receive during a single cycle of treatment.

Female cynomolgus monkeys were infused twice weekly with physiological saline or MORAb-003 at 2.86, 8.55, and 17.10 mg/kg, twice weekly for 4 weeks. The dose volume was 4 ml/kg/hour for all dose groups. At the end of the treatment period, 3 animals in each group were euthanized and necropsied. The remaining two animals in the untreated and 17.1 mg/kg MORAb-003 groups were observed for an additional 28 days before necropsy. The following parameters were evaluated in all animals throughout the study: viability, clinical observations, ophthalmology, electrocardiograms, respiration rates, body weights, food consumption, clinical pathology (pretest and termination), organ weights, macroscopic observations and microscopic pathology. Special attention was given to the tissues previously shown to be stained in the preliminary immunohistochemistry studies. Subsequently, a six-month study was completed, using weekly dosing of 0, 2.9 or 5.7 mg/kg for 24 weeks. A subset of animals was observed for four additional weeks after dosing and prior to necropsy.

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