Balancing Efficacy and Safety of an Anti-DLL4 Antibody through Pharmacokinetic Modulation

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Abstract

Purpose: Although agents targeting Delta-like ligand 4 (DLL4) have shown great promise for angiogenesis-based cancer therapy, findings in recent studies have raised serious safety concerns. To further evaluate the potential for therapeutic targeting of the DLL4 pathway, we pursued a novel strategy to reduce toxicities related to DLL4 inhibition by modulating the pharmacokinetic (PK) properties of an anti-DLL4 antibody.

Experimental Design: The F(ab)’2 fragment of anti-DLL4 antibody (anti-DLL4 F(ab)’) was generated and assessed in efficacy and toxicity studies.

Results: Anti-DLL4 F(ab)’2 enables greater control over the extent and duration of DLL4 inhibition, such that intermittent dosing of anti-DLL4 F(ab)’2 can maintain significant antitumor activity while markedly mitigating known toxicities associated with continuous pathway inhibition.

Conclusions: PK modulation has potentially broad implications for development of antibody-based therapeutics. Our safety studies with anti-DLL4 F(ab)’2 also provide new evidence reinforcing the notion that the DLL4 pathway is extremely sensitive to pharmacologic perturbation, further underscoring the importance of exercising caution to safely harness this potent pathway in humans. Clin Cancer Res; 1–11. ©2015 AACR.

Introduction

Inhibition of tumor angiogenesis has become a prominent strategy in cancer therapy because tumor growth is critically dependent on neovascularization to support ever-increasing metabolic demands. In addition to vascular endothelial growth factor (VEGF; refs. 1–3), members of the Notch signaling pathway, including the NOTCH1 receptor and its Delta-like ligand 4 (DLL4), have been recognized as attractive targets for tumor angiogenesis due to their essential roles in vascular development. DLL4 was initially identified as an endothelium-specific Notch ligand (4–7), and plays a unique role in embryonic and postnatal vascular development, wound healing, as well as tumor angiogenesis (8, 9). Specifically, inhibition of DLL4 signaling during angiogenesis disrupts the dynamic balance between tip and stalk cells (10), resulting in a chaotic vascular network characterized by excessive sprouting and branching, dramatically reduced vessel lumen size, and increased vascular density (11–13). DLL4 neutralizing (anti-DLL4) antibodies have been associated with robust antitumor activity in a wide range of preclinical efficacy models (14–18), likely by promoting nonproductive angiogenesis that impairs tumor microcirculation and induces hypoxia (19, 20). In addition to the potent antitumor activity observed following anti-DLL4 treatment, additive antitumor activity has also been observed in combination with anti-VEGF therapy and/or chemotherapy (14, 16–18, 21). Based on the important role of DLL4 in vascular biology as well as the broad preclinical antitumor activity of anti-DLL4 monoclonal antibodies, several anti-DLL4 molecules are currently being investigated in clinical trials as potential cancer therapeutics (22).

Despite the promise, preclinical studies have raised safety concerns related to inhibiting DLL4. Specifically, treatment of mice, rats, and cynomolgus monkeys with anti-DLL4 resulted in pathologic changes in the liver, including marked atrophy of centrilobular hepatic cords, dilation of centrilobular hepatic sinusoids (sinusoidal dilation), bile duct proliferation, and elevated liver function tests (23). In addition to liver findings, vascular neoplasms occurred in skin, heart, and lungs of male rats after 8 weeks of continuous anti-DLL4 exposure (23). Finally, although vascular neoplasms were not observed in monkeys, dose-dependent, moderate-to-severe acute hemolytic anemia occurred in some animals, leading to mortality in the most severe cases. Clinical trials of the DLL4-targeting antibody OMP-21M18...
Translational Relevance
Several anti-DLL4 molecules are currently being investigated in clinical trials for their potential as cancer therapeutics. Although selective inhibition of DLL4 apparently avoids some known toxicities that have hampered the therapeutic application of Notch inhibition using gamma secretase inhibitors (GSI), safety concerns related to DLL4 inhibition have been raised. Clinical trials using the DLL4-targeting antibody OMP-21M18 have also revealed safety concerns in humans, including grade III asymptomatic hypertension. This manuscript explores a novel strategy to mitigate toxicity while maintaining therapeutic activity in the context of a potent biologic pathway with a relatively narrow therapeutic index, which could have important ramifications beyond developing therapeutics targeting DLL4. In addition, we report new safety findings that further underscore the importance of exercising caution in clinical development of any therapeutics targeting this potent pathway.

have also revealed safety concerns in humans, including grade III asymptomatic hypertension, that were not noted in published preclinical studies (22).

As the outcome of Notch signaling is highly dependent on the context, duration, and strength of pathway activation/inhibition, directly manipulating any of these parameters could offer an opportunity to improve the therapeutic window associated with targeting DLL4. We hypothesized that incomplete or intermittent blockade of DLL4 may have the potential to reduce dose-related liver and vascular toxicities while still maintaining efficacy. We explored a novel strategy of inhibiting DLL4 by reducing the half-life of an anti-DLL4 IgG1 antibody (16, 23), and found that altering the pharmacokinetic (PK) profile by administering a rapidly clearing F(ab')2 antibody fragment allowed more flexible control over the extent and duration of DLL4 inhibition. Accordingly, we demonstrated that pulsatile DLL4 inhibition with an anti-DLL4 F(ab')2 antibody fragment maintained significant antitumor efficacy, yet ameliorated the continuous pathway blockade-associated toxicities of the full-length IgG1 molecule. However, the observation of unexpected safety-related findings further underscores the importance of understanding both the context and duration of NOTCH1 inhibition associated with therapies targeting the DLL4 pathway.

Materials and Methods
Experiments were designed to explore the hypothesis that the extent and duration of DLL4 inhibition is related to both antitumor efficacy and pathway-related toxicity, and that it may be possible to maintain efficacy while reducing toxicity to enhance the therapeutic potential of an anti-DLL4 antibody in humans. To test this hypothesis, we altered the PK profile (i.e., half-life) of an anti-DLL4 neutralizing IgG1 antibody. Efficacy was evaluated in human tumor xenograft mouse models, whereas toxicity was evaluated in normal mice, rats, and monkeys.

All animal studies were conducted in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1985). An Institutional Animal Care and Use Committee (IACUC) approved all animal protocols.

Generation of anti-DLL4 IgG1 lesser affinity variants
The anti-DLL4 IgG1 antibody (YW152F; ref. 16) was subjected to site-directed mutagenesis via alanine replacement of those single amino acid residues in the complementarity determining regions (CDR) that are predicted to be involved in antigen binding.

Production of anti-DLL4 F(ab')2
Anti-DLL4 F(ab')2 was prepared by using pepsin-based solution digestion of the full-length anti-DLL4 parental antibody (YW152F; see Supplemental methods).

Antitumor efficacy studies with xenograft tumor models
The human HM7 colorectal xenograft tumor model was used to assess antitumor efficacy of affinity variants of anti-DLL4 (LM1 and HM6). When the average tumor size reached 150 mm3 in nude female mice, animals were sorted into groups consisting of 8 to 10 mice/group and administered LM1, HM6, or vehicle (phosphate buffered saline, PBS) via i.p. injection at a dose of 10 mg/kg twice per week. The antitumor efficacy of anti-DLL4 F(ab')2 was assessed in the human SW620 colon carcinoma xenograft model in athymic nude female mice and the human Calu-6 lung cancer xenograft model in beige nude female mice. When the average tumor size reached a target size of 100 mm3, animals were sorted into groups consisting of 8 to 10 mice/group and treated intraperitoneally with anti-DLL4 F(ab')2, anti-DLL4 IgG1, or vehicle (PBS). Anti-DLL4 F(ab')2 was administered at various dose levels (cumulative doses of 20, 40, or 80 mg/kg/week) using several dosing regimens (1 on/6 off, 2 on/5 off, or 3 on/4 off), over a duration of 3 weeks. Anti-DLL4 IgG1 was administered at 10 mg/kg/week, once weekly for 3 weeks. Vehicle was also administered once weekly for 3 weeks. In all xenograft studies, tumor volumes and body weights were recorded at regular intervals, and tumor growth was quantitated by caliper measurements. Tumor volume (mm3) was determined by measuring the length (l) and width (w) and calculating the volume (V = lw2/2).

PK analysis of anti-DLL4 antibodies in mice, rats, and cynomolgus monkeys
Female athymic nude mice received a single i.v. dose of anti-DLL4 F(ab')2 (10 or 50 mg/kg; n = 12/group) or anti-DLL4 IgG1 (20 mg/kg; n = 15/group) via the tail vein. Blood samples from three mice per time point at various time points up to 28 days were collected via retrolental bleeds (150 uL of blood collected once per eye prior to terminal sac) and a terminal sample was collected via cardiac stick from each animal in each dose group. Composite serum concentration–time profiles were constructed for PK analysis. In multiple-dose toxicokinetic studies, Sprague Dawley rats received anti-DLL4 F(ab')2 (n = 6 per group) i.v. via the tail vein at 3, 10, 30 or 100 mg/kg (2 doses/week) for a total of 18 doses in 8 weeks and anti-DLL4 IgG1 (n = 3 per gender per group) at 13, 3, 10, or 30 mg/kg (1 dose/week) for a total of nine doses in 8 weeks. Blood samples were collected from each animal via the jugular vein. Composite serum concentration–time profiles were constructed for PK analysis. Female cynomolgus monkeys (n = 5 per group) received multiple doses of anti-DLL4 F(ab')2, i.v. via the
saphenous vein at 5, 15, or 50 mg/kg (1 dose/week) for a total of nine doses in 8 weeks and multiple doses of anti-DLL4 IgG1 (n = 3 per group) at 0.2, 0.8, 3, or 12 mg/kg (1 dose/week) for a total of nine doses in 8 weeks. Blood samples were collected from each animal via the femoral vein and processed to collect serum. Mean serum concentration–time profiles were constructed for PK analysis.

The following PK parameters were estimated using noncompartmental analysis (WinNonlin, version 5.2.1; Pharsight Corporation) based on mouse serum concentration–time profiles: total drug exposure defined as area under the serum concentration–time curve extrapolated to infinity (AUC0–inf), total clearance (CL), and observed maximum serum concentration (Cmax). The following PK parameters were estimated using noncompartmental analysis (WinNonlin, version 5.2.1; Pharsight Corporation) based on rat and monkey serum concentration–time profiles: weekly AUC defined as area under the serum concentration–time curve extrapolated to Day 7 (AUC0–7) and observed maximum serum concentration (Cmax).

A naïve pooled approach was used in mice and rats to provide one estimate for each dose group; whereas in monkeys serum from each animal was analyzed separately and results for each dose group were summarized as mean ± SD. Anti-DLL4 F(ab’)2 or anti-DLL4 IgG1 serum concentrations and antitherapeutic antibody (ATA) responses were assessed using standard ELISAs (see Supplemental methods).

Pilot assessment of anti-DLL4 F(ab’)2 toxicity in rat liver

Experimentally naïve male Sprague Dawley Crl:CD(SD) rats (n = 9/group) were given anti-DLL4 F(ab’)2 i.v. via the tail vein at a total dose of 0 (PBS vehicle), 10, 30, or 100 mg/kg weekly for 8 weeks (2 on/5 off dosing schedule). Males were chosen for pilot evaluations based on the higher incidence of neoplastic lesions seen in males versus females following treatment with anti-DLL4 IgG1 (23). Toxicity evaluation was based on clinical observations, body weights, clinical pathology (clinical chemistry, hematology, and/or coagulation parameters), and macroscopic and microscopic pathology. Serum PK and ATA responses were assessed. Animals were euthanized on Day 59 and organs were collected for microscopic evaluation by a board-certified veterinary pathologist (J.C. Beyer).

Full evaluation of anti-DLL4 F(ab’)2 toxicity profile in rats and cynomolgus monkeys

Experimentally naïve Sprague Dawley Crl:CD(SD) rats (n = 15/sex/group) were given anti-DLL4 F(ab’)2 i.v. via the tail vein at 0 (vehicle), 3, 10, 30, or 100 mg/kg/week (total dose level) on a 2 on/5 off dosing schedule over 8 weeks (e.g., Days 1, 2, 8, 9, 15, 16, 22, 23, 29, 30, 36, 37, 43, 44, 50, 51, 57, and 58; 18 total doses). Vehicle consisted of 20 mmol/L Na2 succinate, 240 mmol/L sucrose, 0.02% polysorbate 20, at pH 5.5. Intermittent assessments of standard toxicologic parameters, including clinical condition, body weight and food consumption, clinical pathology, ophthalmic examinations (using an indirect ophthalmoscope and a slit lamp biomicroscope), and neurologic examinations (24, 25) were conducted. Satellite animals (6/sex/group) were included in the study for the purpose of assessing the kinetics of anti-DLL4 F(ab’)2 exposure; these animals were observed clinically but no other toxicity assessments were conducted. Serum for assessment of ATAs was collected from all animals. Animals were euthanized on Day 59 (terminal necropsy) and 144 (recovery necropsy) and organs were collected for microscopic evaluation by a board-certified veterinary pathologist (J.C. Beyer or G. Cain).

Experimentally naïve cynomolgus monkeys (3–5 years of age, Chinese origin; n = 5/sex/group) were given anti-DLL4 F(ab’)2 i.v. via the saphenous vein at dose levels of 0 (vehicle), 5, 15, or 50 mg/kg once weekly (nine doses total). Vehicle consisted of 20 mmol/L Na2 succinate, 240 mmol/L sucrose, 0.02% polysorbate 20, at pH 5.5. Periodic assessments of standard toxicologic parameters, including clinical condition, body weight, clinical pathology (clinical chemistry, hematology, and/or coagulation parameters), as well as physical, ophthalmic (using an indirect ophthalmoscope and a slit lamp biomicroscope), and neurologic examinations (ref. 26; to assess general neurobehavioral condition and reflexes) were conducted. A subset of animals was implanted with telemetry devices for cardiovascular monitoring; cardiovascular assessments in non-telemetered animals were performed using external leads. Serum PK and ATA assays were performed to confirm exposure to anti-DLL4 F(ab’)2 and assess the antibody response. Animals were euthanized on Day 58 (terminal necropsy) and Day 113 (recovery necropsy) and organs were collected for microscopic evaluation by a board-certified veterinary pathologist (J.C. Beyer or G. Cain). Both the rat and monkey studies were conducted at Covance Laboratories under Good Laboratory Practices (GLP).

Results

Reducing DLL4 binding is not adequate to separate efficacy from toxicity in mice

We reasoned that tumor vasculature and normal liver vasculature might be differentially sensitive to DLL4 inhibition, such that partial DLL4 blockade could provide an opportunity to separate antitumor efficacy from liver toxicity and improve the therapeutic window. To this end, we explored a strategy of reducing antibody binding affinity through alanine-directed mutagenesis in the antibody CDRs to generate a panel of antibody variants with reduced binding to DLL4 on endothelial cells relative to parental antibody (Supplementary Fig. S1). The reduced-binding antibodies were evaluated for antitumor efficacy in an HM7 colon cancer xenograft model and for induction of liver toxicity in nude mice. The results of these in vivo studies defined two general classes of antibody variants, exemplified by candidate molecules LM1 and HM6 (Fig. 1). Specifically, the reduced binding of LM1 relative to the parental antibody was associated with attenuated antitumor activity (Fig. 1A) without notable attenuation of liver toxicity (Fig. 1B) after 3 weeks of continued exposure. Compared with LM1, HM6 had even lower binding affinity and resulted in a complete loss of both antitumor activity and liver toxicity. Reducing antibody binding therefore failed to yield a molecule with the favorable properties of sustained antitumor activity combined with reduced liver toxicity.

Qualitative characterization of activity and pharmacokinetics of anti-DLL4 F(ab’)2

The anti-DLL4 F(ab’)2 antibody fragment retains bivalent CDRs identical to the parental antibody, and is expected to have equivalent target binding capacity. To confirm that generation of the F(ab’)2 antibody fragment did not impact activity, we first tested the in vitro potency of the F(ab’)2, relative to the parental IgG1 in a three-dimensional angiogenic sprouting assay (27). As anticipated, anti-DLL4 F(ab’)2 caused a marked increase in angiogenic sprouting that was comparable to that observed following
treatment with anti-DLL4 IgG1 (Supplementary Fig. S2A). We also tested the activity of anti-DLL4 F(ab')2 in vivo by evaluating early postnatal mouse retina, which develops a stereotypic vascular pattern in a well-defined sequence (28–30). Consistent with the in vitro results, anti-DLL4 F(ab')2 caused excessive angiogenic sprouting in the mouse retina that was qualitatively indistinguishable in terms of vascular morphology from that observed after anti-DLL4 IgG1 treatment (Supplementary Fig. S2B). Although the in vitro activity of some antibodies may involve Fc-mediated effector function, these results, together with the subsequent tumor efficacy studies (detailed below), demonstrate that the biologic activity of anti-DLL4 antibodies is independent of effector function and therefore supports the potential utility of an F (ab')2 antibody fragment to evaluate intermittent DLL4 pathway blockade. The pharmacokinetics of the F(ab')2 and IgG1 antibodies were evaluated in athymic nude mice following administration of a single i.v. bolus dose. The exposure profile of both molecules was also evaluated in repeat-dose toxicity studies in rats and cynomolgus monkeys. In mice, at a dose of 10 mg/kg, anti-DLL4 F(ab')2 had a clearance of 362 mL/day/kg compared with 8 mL/day/kg for the full-length IgG1 (Supplementary Table S1; ref. 31). Similarly, in rats and cynomolgus monkeys, anti-DLL4 F(ab')2 was cleared much more rapidly relative to the full-length IgG1 (Fig. 2, Supplementary Fig. S3; see Supplementary Table S2 for a full listing of PK parameters). These results confirmed that the anti-DLL4 F(ab')2 molecule was an appropriate tool to assess the impact of intermittent DLL4 pathway inhibition on both efficacy and toxicity.

Figure 1.
Reducing binding affinity of anti-DLL4 IgG1 does not separate antitumor efficacy and liver toxicity in mice. A, robust antitumor efficacy was observed in a human HM7 xenograft model with anti-DLL4 IgG1 (YW152F), whereas lower affinity variants showed attenuated (LM1) or no efficacy (HM6.1) when administered twice per week i.p. at 10 mg/kg. Tumor volumes are presented as mean ± SEM (n = 8–10/group). Two-tailed unpaired t test was used to calculate P values at Day 11. B, mice given anti-DLL4 IgG1 for 3 weeks at 30 mg/kg/week i.p. had marked sinusoidal dilation of the liver, as shown by hematoxylin and eosin (H&E) staining. Lower affinity variants showed either a similar severity of liver pathology (LM1) relative to the IgG1 molecule, or appeared histologically normal and similar to control-treated animals (HM6) following an equivalent dosing regimen. Scale bar = 500 μm.

Figure 2.
PK profiles of anti-DLL4 IgG1 and F(ab')2 in mouse and monkey. A, anti-DLL4 IgG1 serum concentration–time data in athymic nude mice; B, anti-DLL4 F(ab')2 serum concentration–time data in athymic nude mice; C, anti-DLL4 IgG1 average serum concentration–time data in cynomolgus monkeys; D, anti-DLL4 F(ab')2 average serum concentration–time data in cynomolgus monkeys.
Efficacy of anti-DLL4 F(ab')2

Anti-DLL4 F(ab')2 showed significant antitumor activity across all treatment groups in a human SW620 colon carcinoma xenograft tumor model, although the extent of antitumor activity was dependent on dose and dosing schedule (Fig. 3A). Using the 2 on/5 off dosing schedule, a dose-dependent antitumor effect was observed when mice were given a cumulative weekly dose of 20, 40, or 80 mg/kg. The duration of DLL4 inhibition also played a role in the extent of activity, with the strongest F(ab')2 antitumor effect observed with the 3 on/4 off dosing schedule, followed by slight but dose-dependent reductions in efficacy using the 2 on/5 off or the 1 on/6 off schedule despite administration of an equivalent total dose of 40 mg/kg/week. Continuous DLL4 inhibition (10 mg/kg/week of the full-length anti-DLL4 IgG1) showed the greatest level of tumor growth inhibition. Similar observations of robust antitumor activity of the F(ab')2 were also noted in a Calu6 lung adenocarcinoma xenograft tumor model (Fig. 3B). These results indicated that pulsatile anti-DLL4 inhibition was sufficient to elicit robust antitumor activity and confirmed the suitability of the 2 on/5 off dosing regimen for further evaluation.

Figure 3.
Anti-DLL4 F(ab')2 shows antitumor efficacy in human xenograft tumor models. Anti-DLL4 F(ab')2 was administered at various dose levels and dosing schedules as indicated. Results from the (A) SW620 human colon carcinoma xenograft tumor model and the (B) Calu6 lung adenocarcinoma xenograft tumor model indicated a relationship between dosing duration and anti-DLL4 F(ab')2 antitumor activity, such that a longer duration of exposure was generally more efficacious relative to the same cumulative dose over the dosing window. A treatment arm with the original anti-DLL4 IgG1 antibody (YW152F) administered at 10 mg/kg/week (i.p.) was also included as a positive control for maximum pathway inhibition and antitumor activity. Mean tumor volumes ± SEM are presented (n = 8–10 mice/group). Two-tailed unpaired t test was used to calculate P values, which are presented in a table for each tumor study.
Mitigating toxicity with anti-DLL4 F(ab')2 in mice and rats

To address whether intermittent blockade of the DLL4 pathway might ameliorate the toxicity associated with continuous pathway inhibition, we first evaluated the effects of anti-DLL4 F(ab')2 on DLL4 pathway-related liver gene expression and histopathology in mice. Marked upregulation of DLL4 blockade-related gene expression occurred in liver, as previously reported by Yan and colleagues (ref. 23; Supplementary Fig. S4A and S4B). Despite a similar cumulative weekly dose of anti-DLL4 F(ab')2, significant attenuation of DLL4 pathway-related gene expression occurred in mice treated on an intermittent dosing schedule (100 mg/kg/week given on the 2 on/5 off schedule). Liver gene expression levels after an 18-day recovery period were comparable between control and anti-DLL4 F(ab')2-treated mice regardless of dosing regimen; however, gene expression increases were sustained in anti-DLL4 IgG1-treated mice likely due to slower systemic clearance of the IgG1 antibody compared with the F(ab')2 (Supplementary Table S1). The nature and severity of hepatic histopathological changes corresponded with altered gene expression in mouse livers for all treatment groups at terminal and recovery time points (Supplementary Fig. S4C). Intermittent dosing of anti-DLL4 F(ab')2 mitigated the effect of DLL4 inhibition in the mouse liver. Furthermore, changes in liver gene expression and microscopic findings were reversible after antibody clearance.

To extend and confirm findings indicating schedule-dependent amelioration of liver toxicity by anti-DLL4 F(ab')2 administration in the mouse, two follow-up pilot toxicity studies were conducted in rats. Studies in rats were important for toxicity screening because rats showed the highest sensitivity to anti-DLL4 IgG1-mediated liver histopathological changes among all nonclinical species evaluated (mouse, rat, and monkey), and also demonstrated the potential for dose-dependent vascular neoplasia after treatment with anti-DLL4 IgG1 (23). In both studies, male Sprague Dawley rats were dosed i.v. with anti-DLL4 F(ab')2 at 0, 10, 30, or 100 mg/kg weekly (2 on/5 off schedule) for 8 weeks (18 doses; n = 9/group); the second confirmatory study also included an 8-week treatment-free recovery period (n = 9/group for both dosing and recovery periods) to evaluate the reversibility of any potential anti-DLL4 F(ab')2-induced lesions. These studies were conducted only in males because vascular neoplasms following IgG1 treatment were observed only in males, although liver toxicity did not show a difference between sexes. Representative results from the second F(ab')2, pilot study are shown in Fig. 4B and D, as the toxicity and exposure profiles of anti-DLL4 F(ab')2-repeated results from the first pilot study. For comparison, the IgG1 rat study results (Fig. 4A and C) are shown with pooled data from males and females, as there were no differences between sexes for liver parameters. Similar to the mouse, the clearance of anti-DLL4 F(ab')2 in rats was much faster than the full-length IgG1 (Supplementary Table S2; Supplementary Fig. S3).

As anticipated based on previous experience with anti-DLL4 IgG1, rats treated with anti-DLL4 F(ab')2 at 30 and 100 mg/kg/week (i.e., 15 or 50 mg/kg/day on the 2 on/5 off schedule) had changes in serum chemistry parameters after 8 weeks of dosing, including minimal to mild increases in serum ALT (Fig. 4B), aspartate aminotransferase, alkaline phosphatase (ALP), total bilirubin, total bile acids, and cholesterol. Corresponding histologic changes in the liver included sinusoidal dilation at all dose levels, as well as hepatocellular necrosis, mixed leukocyte inflammation, and bile duct hyperplasia (Fig. 4D). Notably, the severity and incidence of hepatotoxicity (clinical and anatomic pathology) was reduced relative to that observed with anti-DLL4 IgG1 at equivalent exposure levels (i.e., 3 mg/kg/week IgG1 vs. 10 mg/kg/week F(ab')2; or 30 mg/kg/week IgG1 vs. 100 mg/kg/week F(ab')2), indicative of a shift in the overall toxicity dose–response (Fig. 4B–D, Supplementary Table S2). Evidence of reversibility of F(ab')2-associated clinical and anatomic pathology changes (sinusoidal dilation/hepatocyte loss) was present, with the exception of nonreversible biliary hyperplasia and fibrosis at a dose of 100 mg/kg/week.

Importantly, cutaneous vascular neoplasia was not present in rats at any F(ab')2 dose level, further differentiating the F(ab')2 toxicity profile from that of the IgG1 molecule in which lesions were present at doses as low as 3 mg/kg/week and increased in incidence at higher dose levels (Supplementary Table S3). Together, these results supported the potential for an improved therapeutic window and reduced toxicity profile with anti-DLL4 F(ab')2 relative to anti-DLL4 IgG1, enabling a path forward for additional characterization of the anti-DLL4 F(ab')2 molecule.

Novel toxicity findings associated with DLL4 pathway inhibition

To extend the observations in mice and rats suggesting the potential for an improved toxicity profile for anti-DLL4 F(ab')2, and to support future clinical studies, definitive (GLP) toxicology studies were conducted in rats and cynomolgus monkeys. Rats were dosed i.v. with anti-DLL4 F(ab')2 at 0, 3, 10, 30, or 100 mg/kg/week for 8 weeks, using the same 2 on/5 off dosing schedule as in the mouse efficacy and pilot rat toxicity studies. To account for distinct PK exposure profiles between species (Fig. 2; Supplementary Table S2), monkeys were dosed once weekly for 8 weeks with anti-DLL4 F(ab')2 i.v. at 0, 10, 30, and 100 mg/kg/week. Both studies included an 8-week treatment-free recovery period to evaluate the reversibility of any drug-induced findings. Additionally, the dosing regimens were designed to produce systemic drug levels in both species that overlapped with exposures previously obtained from the anti-DLL4 IgG1 molecule, thereby enabling a comparison of the relative toxicity profiles between these two molecules (see Fig. 4).

Consistent with effects observed in the rat pilot toxicity studies, shifts in the dose–toxicity relationship of the F(ab')2 molecule relative to the IgG1 molecule were observed in both rats and cynomolgus monkeys. Higher dose levels of the F(ab')2 were necessary to elicit the same incidence and severity of IgG1-mediated changes, despite approximately equivalent AUC (Fig. 5A and B) and equivalent or higher Cmax (Supplementary Table S2). Together with the results of the rat pilot toxicity studies (Fig. 4; Supplementary Table S2), these observations suggest that maintenance of anti-DLL4 concentrations over a threshold concentration may be the driver of toxicity, because the faster clearance of F(ab')2 allows the serum concentrations to drop to a much lower level within the dosing period compared with the full-length IgG1. For example, the incidence and severity of anemia in monkeys were decreased with the F(ab')2 relative to the IgG1 molecule (Fig. 5). Observations of an attenuated toxicity profile for the F(ab')2 relative to the full-length IgG1 were consistent across species (mouse, rat, and cynomolgus monkey) and indicate that intermittent inhibition of DLL4 mitigates toxicities associated with continuous DLL4 blockade, likely due to partial recovery of pathway inhibition between dose administrations.
Figure 4.
Reduced toxicity of anti-DLL4 F(ab')2 relative to IgG1 in rat liver. Comparison of liver enzyme changes and incidence of liver lesions revealed an amelioration of toxicity when dosing with anti-DLL4 F(ab')2 relative to the IgG1 at comparable exposures (AUC0–7). A and B, liver enzyme (ALT) profiles of individual rats are shown over an 8-week dosing period with anti-DLL4 IgG1 (A) or anti-DLL4 F(ab')2 (B); bold lines, the group mean with points at the actual measurement times. For both the IgG1 (n = 15/sex/group) and F(ab')2 (n = 18 males/group) datasets, dosing and recovery animals were pooled across the dosing interval; for the IgG1, data were also pooled across sex as there was no apparent sex difference in liver toxicity. Only males were used in the F(ab')2 study. Scaling of the y-axis is on a log2 scale so that each axis tick represents a doubling of the ALT values, with labeling on the natural scale to facilitate interpretation. C and D, the frequency and severity of major histologic findings in the liver was dose-dependent for both anti-DLL4 F(ab')2 and IgG1 following 8 weeks of IgG1 administration. However, relative to the IgG1 liver toxicity profile (n = 10/sex/group) (C), dosing F(ab')2 on a 2 on/5 off dosing schedule (n = 9 males/group) revealed amelioration of severe liver pathologies (BDH, fibrosis, necrosis) (D). SNDIL, sinusoidal dilation; BDH, bile duct hyperplasia.
Despite evidence of amelioration of known anti-DLL4–related toxicities, the 8-week F(ab\textsubscript{0})\textsubscript{2} toxicity studies in rat and monkey revealed unexpected toxicities that were distinct from those observed following administration of the full-length IgG1 molecule. Specifically, anti-DLL4 F(ab\textsubscript{0})\textsubscript{2} administration was associated with a dose-related increase in the incidence and severity of vascular changes in the heart and lung, including endothelial cell proliferation on the right side of the heart and proliferative pulmonary artery mural/adventitial changes (rats only), intimal vacuolation in the pulmonary artery at the base of the heart (monkeys only), and increased cellularity of arteries in the lung (both species; Fig. 6 and Supplementary Fig. S5A and S5B). Additionally, pulmonary artery mural basophilia was present in the heart and lung in both species. After an 8-week recovery period, the incidence and severity of cardiac and pulmonary findings had decreased in both species; however, pulmonary arterial adventitial fibrosis was present in rats and was considered likely sequelae of adventitial proliferative changes present after the dosing period (Supplementary Fig. S5C and S5D). The nature of the vascular changes present in the pulmonary artery and right side heart tissue was consistent with likely pulmonary arterial hypertension (PAH).

Details of the procedures used to develop Supplementary data are described in Supplemental Methods.

Discussion

The well-established role of DLL4/NOTCH1 in tumor angiogenesis and the potent efficacy associated with pathway inhibition across numerous preclinical tumor models has advanced several DLL4 neutralizing antibodies (OMP-21M18, REGN421, and MEDI0639) into clinical studies in various solid tumor settings (22, 32). We previously reported that chronic administration of a full-length anti-DLL4 IgG1 antibody was associated with induction of vascular neoplasms in rats as well as pathologic changes in the liver in rats and monkeys (23). The likely relationship of on-target liver toxicity, in addition to the overall severity and inability to monitor lesions produced, raised major safety concerns around inhibition of the DLL4/Notch pathway (23, 32). Based on the promise of DLL4 as a therapeutic target, we investigated potential methods for improving the safety profile of anti-DLL4 therapies.

Initial studies with affinity variants generated from the parental anti-DLL4 IgG1 antibody revealed that reducing affinity alone did not provide adequate separation of efficacy and toxicity, at least in the context of the currently studied antibody. Considering lessons learned from the preclinical and clinical development of γ-secretase inhibitors, in which an intermittent dosing strategy was successfully leveraged to maximize therapeutic potential while mitigating safety concerns (33), we generated a F(ab\textsubscript{0})\textsubscript{2} antibody fragment of anti-DLL4 that has a substantially shortened half-life in mice, rats, and monkeys (ref. 34; Supplementary Tables S1 and S2). Studies in human tumor xenograft models confirmed that the F(ab\textsubscript{0})\textsubscript{2} antibody fragment retained antitumor efficacy in vivo. We further demonstrated that the extent of tumor growth inhibition depended on maintenance of anti-DLL4 F(ab\textsubscript{0})\textsubscript{2} serum concentrations above a threshold exposure level over a period of time (Fig. 3), due to a direct relationship between serum anti-DLL4 antibody concentration and duration of pathway inhibition.

Unlike the reduced affinity variants of the anti-DLL4 IgG1, the F(ab\textsubscript{0})\textsubscript{2} antibody fragment was able to maintain antitumor efficacy and mitigated the primary safety concerns identified with the original IgG1 molecule, indicating that the minimum exposure

Figure 5.
Shift in toxicity dose–response in monkeys with anti-DLL4 F(ab\textsubscript{0})\textsubscript{2}. In monkeys, the most severe DLL4-related toxicity was anemia. The incidence and severity of anemia observed following 8 weeks of anti-DLL4 IgG1 (n = 5/sex/group) (A) was mitigated by dosing with F(ab\textsubscript{0})\textsubscript{2} (n = 5/sex/group) (B) at overlapping or higher exposure levels. Bold lines, group mean with points at actual measurement times. Dashed lines, animals that required unscheduled euthanasia due to severe anemia.
duration required for antitumor activity was less than that required to elicit toxicity. Specifically, the F(ab')2 molecule appeared to result in a shift in the toxicity–exposure relationship relative to the IgG1 molecule, as evidenced by a reduction in the incidence and severity of serum liver enzyme elevations and liver histopathology findings in rats and monkeys (Fig. 4), a reduction in the incidence and severity of anemia in monkeys (Fig. 5), as well as a complete mitigation of vascular neoplasms in rats (Supplementary Table S3). These results indicate that alterations in the exposure profile of an antibody (e.g., by using a F(ab')2) may be a valuable strategy to mitigate toxicity while maintaining therapeutic activity in the context of a potent biologic pathway with a relatively narrow therapeutic index.

Despite the mitigation of the well-known adverse effects, we observed novel toxicities after repeated administration of anti-DLL4 F(ab')2 over 8 weeks including right heart endothelial cell proliferative changes and proliferative pulmonary artery mural/adventitial changes (rats only), intimal pulmonary artery vacuolation (monkeys only), and pulmonary artery basophilia in the heart and lung (both species). These right heart and pulmonary arterial vascular changes are consistent with the histologic sequelae of drug-induced PAH (refs. 35, 36; Fig. 6); notably, these findings were not present after administration of the full-length anti-DLL4 IgG1. The cardiac and pulmonary vascular changes that occurred after administering the F(ab')2 antibody fragment further underscore the importance of context-dependent outcomes associated with perturbation of DLL4/NOTCH1 signaling within different tissues.

Findings accumulated from our experience with anti-DLL4 antibodies indicate that inhibition of the DLL4 pathway is broadly associated with adverse effects in at least two major organ systems: the liver and cardiopulmonary systems, specifically, the right heart and pulmonary arterial vasculature. Histopathological changes in the heart and lung were not accompanied by overt...
changes in heart rate or blood pressure in telemetry-instrumented monkeys, suggesting that these changes are likely to be poorly monitorable in the clinic. Independent preclinical and clinical investigations using other anti-DLL4 antibodies indicate that the constellation of changes in the liver, skin, and cardiopulmonary systems may represent a class-effect of DLL4 inhibition. For instance, with respect to changes in the cardiopulmonary system, an anti-DLL4 antibody (MEDI0639) also evaluated in monkeys was associated with pathologic changes in the heart and lung, as indicated by elevations in blood pressure, heart rate, and C-reactive protein, and observations of heart failure (37). In humans, another anti-DLL4 antibody (OMP-21M18) has been associated with grade III asymptomatic hypertension in approximately 28% of phase I patients (22). In addition, an anti-DLL4 antibody (REGN421) reported hypertension as well as grade II and III pulmonary hypertension in 14% of patients in a phase I trial (38). Therefore, the totality of the effects observed over a range of anti-DLL4 neutralizing antibodies indicates a liability of DLL4 inhibition in the cardiopulmonary system that must translate to a broad safety risk in humans. Of particular concern, our data indicate clear species differences in sensitivity for particular DLL4-mediated toxicities (e.g., rats appear most sensitive to effects on liver, whereas monkeys are most sensitive to effects on RBC parameters). This species-specific heterogeneity of response to DLL4/NOTCH1 pathway inhibition complicates prediction of the most likely or severe safety concerns in humans.

Taken together, by utilizing a F(ab’)2 antibody fragment against DLL4 we have demonstrated a unique approach to antibody drug development that may prove advantageous for widening or improving a narrow therapeutic index, particularly if toxicities appear related to chronic pathway inhibition. Importantly, our data provide further evidence that the DLL4 pathway is extremely sensitive to pharmacologic perturbation and that toxicities may be difficult to accurately model in preclinical species and/or monitor in the clinic. Therefore, caution must be exercised to safely harness this potent pathway to treat cancer.

**Disclosure of Potential Conflicts of Interest**
A.V. Kamath has ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.

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