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Development of XPA067.06, a potent high affinity human anti-gastrin monoclonal antibody

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ABSTRACT

The peptide hormone gastrin is a key factor in regulation of gastric acid secretion. It has also been implicated in the development or maintenance of various types of cancer, such as pancreatic and stomach carcinoma. Inhibition of gastrin activity has potential for therapeutic use as a suppressor of acid secretion as well as an inhibitor of gastrin-responsive tumors. XPA067.06 is an affinity matured, 30 pM fully human anti-gastrin monoclonal antibody that was generated. The antibody was tested in a mouse gastric pH model to determine its effect on acid secretion. In this model, animals were treated with human gastrin, XPA067.06, and H2R or M1 receptor antagonists. Gastric fluid was collected and acid output was measured as a function of pH. XPA067.06 was shown to significantly inhibit gastrin-17-stimulated acid output for at least 48 h. These results demonstrate that XPA067.06 effectively binds and neutralizes human gastrin-17 in vivo with rapid onset and prolonged duration of efficacy.

1. Introduction

Gastrin was first discovered by Edkins in 1905 [1]. It is produced as a 101 amino acid precursor pre-pro-gastrin, which is cleaved to generate several different proteins of varying length [2]. Additional post-translational modifications include glycine addition and amidation. The three most important species in terms of biologic activity and levels detectable in serum and plasma are a 34 amino acid form (amidated G34); a 17 amino acid form (amidated G17), and a precursor to G17 with an additional glycine at the C-terminal end (glycine-extended G17, or Gly-G17). Conventionally, gastrin refers to G17. Glycine-extended gastrin has little effect on acid secretion, but may potentiate the effect of amidated gastrin. It can stimulate colonic proliferation and accelerate the development of colon cancer [3]. The longer (gastrin-52 and gastrin-71) and shorter (gastrin-14 and gastrin-6) secondary gastrins are also released into plasma, but in low concentrations [4].

The regulation and mechanisms of gastric acid secretion are complex, including neural, hormonal, paracrine, autocrine, and intracellular pathways [5]. The main stimulants of acid secretion are the hormone gastrin, the paracrine amine histamine and the proton pump H^+K^+-adenosine triphosphatase (H^+K^+-ATPase) [4]. Gastrin induces acid secretion by activating cholecystokinin-2 (CCK-2; formerly CCK-B or CCK-B/gastrin) receptors, while histamine acts via H_2 receptors.

Gastrin stimulates acid secretion directly and, more importantly, indirectly by releasing histamine [6]. Gastrin knockout and CCK-2 receptor-null mice fail to respond appropriately to exogenously administered gastrin and his-

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tamine, whereas insulin–gastrin transgenic mice that overexpress amidated gastrin have increased acid secretion [7,8]. These studies suggest that gastrin is essential for the functional maturation of the acid secretory system.

In addition to its role in regulating acid secretion, gastrin acts as a growth factor in several cancer types including gastric, pancreatic, and colorectal carcinoma [9–11]. Expression of gastrin and its receptors has been detected in primary tumors taken from cancer patients [12–16]. Gastrin is believed to act as growth factor through autocrine, paracrine and endocrine stimulation of tumor cells [17]. The therapeutic approach of disrupting the gastrin-mediated mitogenesis of cancer cells has been tried in the clinic using small molecule antagonists to the gastrin receptor [18].

The approach of stimulating anti-gastrin antibodies as a treatment of gastrin mediated diseases has been tested in clinical trials of the vaccine Gastrimmune™ (G17DT). G17DT is a form of gastrin consisting of the first nine N-terminal amino acids of human gastrin linked through a peptide spacer to the immunogenic carrier, diphtheria toxoid. Immunization with G17DT elicits the production of neutralizing antibodies to the amidated and glycine-extended forms of G17, which block gastrin from binding to the CCK-2 receptors, resulting in an inhibition of gastrin-stimulated growth of colon cancer tumors [19]. The inhibition of acid secretion via G17DT-induced anti-gastrin antibodies was observed in the pig and rat models [20,21]. A phase III trial in patients with pancreatic cancer confirmed improved survival of patients in the G17DT group through an intention-to-treat analysis [22]. The therapeutic strategy using G17DT vaccine, however, has some significant limitations. Many patients are immunocompromised, resulting in an inadequate response to the vaccine or completely fail to develop a detectable antibody titer. It has also been observed that the time to build an adequate anti-gastrin titer in responder patients is from 4 to 12 weeks, effectively resulting in a delay of treatment. Additionally, G17DT-specific abscesses occur at the site of IM injection.

An opportunity to overcome these limitations is offered through the development of a potent anti-gastrin antibody for passive immunotherapy. High affinity human anti-gastrin antibody such as XPA067.06 described in this report would provide immediate coverage upon treatment of all patients and potentially offers an improved safety profile. To determine the efficacy of XPA067.06 in vivo, gastric pH mouse models were established to measure the neutralization of human G-17 (h-G17). In this report we demonstrate that XPA067.06 neutralizes human gastrin and reduces h-G17-stimulated gastric acid secretion in vivo.

2. Materials and methods

2.1. Peptides used for antibody selections and characterization

- *9-mer:* Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Lys-linker-biotin.
- *14-mer:* Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Lys-linker-biotin.
- *17-mer:* Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-Lys-linker-biotin.

Peptides were synthesized by Anaspec, Inc. (San Jose, CA).

2.2. Isolating G17-specific antibodies

Selection of target-specific antibody from phage display was carried out according to methods described by Marks and Bradbury [23]. Briefly, each library was incubated with 100 pmoles of the biotinylated 9-mer peptide at room temperature for 1 h, followed by the addition of 100 μl of Streptavidin beads in suspension (DYNABEADS® M-280 Streptavidin, Invitrogen, Carlsbad, CA). Non-specific phage were removed by washing the beads with wash buffer (PBS +5% milk), then the bound phage were eluted with 0.5 ml of 100 nM Triethylamine (TEA) and immediately neutralized by addition of an equal volume of 1 M TRIS–Cl pH 7.4. Eluted phage were used to infect TG1 *E. coli* cells growing in logarithmic phase, and phagemid was rescued as described [23]. Selection was repeated for a total of three rounds. Single colonies obtained from TG1 cells infected with phage eluted from the third round of panning were screened for binding activity in an ELISA assay. Microcultures in 96-well plates were grown to an OD_{600} = 0.6, induced with 1 mM IPTG and incubated overnight in a shaker incubator at 30 °C. Bacteria were pelleted, resuspended in 50 μL PPB (Teknova, Hollister, CA), and periplasmic extracts prepared by addition of 75 μL of sterile water, followed by shaking at 4 °C for 50 min. The periplasmic extracts were tested for antibody binding activity to biotinylated gastrin immobilized on Streptavidin microplates (REACT-BIND™ Streptavidin HBC, Pierce, Rockford, IL) following the standard ELISA protocol provided by the microplate manufacturer. The ELISA was detected with anti-myc HRP.

2.3. Off-rate ranking of antibody fragments

The relative off-rates of the ELISA positive clones were analyzed using a BIACORE® 2000 as follows. Biotinylated 9-mer and 14-mer peptides were injected over a Streptavidin sensor chip (BIACORE®), resulting in immobilization of 1–2 Response Units (RU). Periplasmic extracts containing the antibody fragments were 0.22 μm-filtered using 96-well filter plates (Millipore, Inc., Billerica, MA) then injected over the chip, resulting in the binding of up to 1500 RU. Data was analyzed using BiaEvaluation (Biacore®) and Scrubber (BioLogics, Inc., Manassas, VI) software. Samples that bound to streptavidin alone or resulted in less than 20 RU of peptide-specific binding were eliminated from the analysis. Relative dissociation rates from both peptides were ranked for all samples within each 96-well plate. In most cases the ranking was consistent for both peptides.

2.4. Converting antibody candidates identified by phage display to Fc fusion proteins and IgG

To convert antibody fragments to scFv-Fc as a surrogate IgG for further characterization, scFv were PCR amplified from the phagemid vector and cloned into pSCF (XOMA; Berkeley, CA) expressing the gamma 2 Fc region genes.

To convert the lead candidate antibody fragments to IgG, the variable regions of both heavy and light chains were cloned into a mammalian expression vector encoding either the kappa (κ) or lambda (λ) light chain and the gamma-2 (γ2) heavy chain constant region genes. ScFv-Fc and IgGs were transiently expressed in 293E cells as described in Handa [24]. Supernatant from transfected cells was harvested at day 6 of culture and antibody was purified using protein A chromatography following manufacturer protocols (Protein A Sepharose™; Amersham Biosciences, Piscataway, NJ).

2.5. Kinetic analysis and off-rate ranking of anti-gastrin scFv-Fc fusions and IgG antibodies

Kinetic analyses of anti-gastrin antibodies were carried out using a Biacore® 2000. Biotinylated 17-mer peptide was immobilized on flowcells 2, 3, and 4 of a Streptavadin sensor chip by injecting 10 μL of peptide diluted to 2.5, 5, and 10 ng/mL, respectively, resulting in a surface density of 2–6 RU. Flowcell 1 was left blank as a reference flow cell (streptavidin surface alone).

Kinetic ranking of affinity-matured scFv-Fc fusions was performed as a secondary screening step. The Fc fusions were captured by protein A/G (Pierce Biotechnology, Rockford, IL) that had been amine-coupled to a CM5 sensor chip. To produce an adequate signal for analysis, the biotinylated 17-mer peptide was incubated with 100-fold molar excess of NeutrAvidin™ biotin binding protein (Pierce, Inc., Rockford, IL). This strategy resulted in a higher molecular weight analyte while avoiding multivalent binding to the tetravalent NeutrAvidin™. The scFv-Fc fusions were diluted to 1 μg/mL in HBS-Ep running buffer and injected over flowcell 2. An irrelevant scFv-fc fusion was captured on flowcell 1. A single concentration (40 nM) of the gastin/NeutrAvidin™ was injected over the captured antibody, resulting in the binding of 100–400 RU. Dissociation rates were calculated using BiaEval.

Kinetic analysis of the anti-gastrin IgGs was conducted using six 3-fold serial dilutions of each antibody in running buffer (HBS-EP). Each dilution was injected in triplicate in random order. Buffer injections were evenly distributed throughout the run. Samples were injected over the 17-mer peptide of human gastrin immobilized on a streptavidin sensor chip as described above (approximately 2 RU of peptide, R_{max} ~50 RU). The analyte injections were double-referenced against the blank flowcells and buffer injections. Data were analyzed with the BiaEvaluation Biacore®. Sensorgrams were fit utilizing a bivalent analyte model.

2.6. AGS-TR calcium flux assay

AGS human gastric adenocarcinoma cells stably transfected with CCK-2 receptor (AGS-TR, generated at Aphton Corp., Woodlands, CA) were grown to approximately 60–70% confluence in RPMI 1640 growth medium containing 10% fetal bovine serum and 0.4 mg/ml G418 antibiotic (Invitrogen, Carlsbad, CA), detached using non-enzymatic dissociation buffer (Invitrogen, Carlsbad, CA) and loaded with 4 μM Fluo-3 AM and 10 μM Fura Red AM (Invitrogen, Carlsbad, CA) in RPMI 1640 with 2% FBS (R2) for 45 min at room temperature on a laboratory shaker. During dye loading the cells were shielded from light. Loaded cells were washed 1× and resuspended in R2 to a final density of 10^6 cells/mL. After the wash step, loaded cells were stored on ice for 30–60 min prior to analysis.

For flow cytometry analysis, 0.5 mL of dye-loaded cells was transferred to a 2.5 mL test tube, which was then placed in a Time Zero system (Cytek Development, Fremont, CA) sample module. The temperature in the sample module was maintained at 37 °C using a heated circulating water bath. The samples were equilibrated at 37 °C for 5 min prior to analysis by flow cytometry. Concurrent with sample warming, 10× stimulus mixture (recombinant h-G17, Sigma, St. Louis, MO, with or without antibody) was warmed to 37 °C on a heating block. For gastrin neutralization testing, antibody and h-G17 were each diluted to 20× final concentration in loading medium and mixed 1:1. Antibody and h-G17 were then incubated over ice for 15–30 min prior to initiation of analysis. Approximately 10 s of data were acquired prior to injection of 50 μL of 10× stimulus mixture through the time zero injection port. Boost pressure was simultaneously applied as stimulus was injected. Data were then acquired for an additional 40 s. Medium and recombinant G17 controls were run at the beginning and end of each experiment to ensure that the calcium flux response of the AGS-TR cell line remained constant throughout.

2.7. Projected anti-gastrin/gastrin PK/PD model

The expected pharmacokinetics and pharmacodynamics (PK/PD) of a monoclonal antibody (mAb) with 5 nM affinity for gastrin was developed to determine the required weekly dose of mAb needed to reduce the degree of gastrin's binding to its receptor. The PK/PD model was developed based on known pharmacokinetics of human antibodies in humans without target, literature values of maximal gastrin levels (0.15 ng/mL) [25], the half-life of gastrin (15 min) [26], and the affinity of gastrin for its natural receptor, CCK-B, on mucosal/cancer cells (2–6 nM) [10]. Typical rate constants for ligand–receptor binding were obtained from the cytokine literature [27]. The model includes a mechanism of clearance of the Ab–gastrin complex, assumed to be 10 times faster than free antibody. The PK/PD model, diagrammed in Fig. 4A, uses best estimate values for the parameters.

2.8. Affinity maturation of anti-gastrin IgG1 antibodies

Affinity maturation was carried out for clone XPA067 as follows. Libraries of antibodies were produced by random mutagenesis of the following regions: V_H and V_L CDR1 and CDR3 and V_H CDR2. The libraries were constructed as described [28]. Each CDR3 was randomized in two blocks of 6 amino acids in order to cover the entire CDR, producing libraries H3B1 (N terminal block of 6 aa VH CDR3), H3B2 (C terminal block of 6 aa in VH CDR3), L3B1 (N terminal block of 6 aa in VL CDR3) and L3B2 (C terminal block of 6 aa in VL CDR3). V_H CDR1 was randomized including the vernier residue at position 30 [29,30] to produce library H1. V_H CDR2 was randomized at residues 50, 52, 53, 54, 56 and 58, to produce library H2. V_L CDR1 was randomized at residues 27A, 27B, 27C, 29, 31 and 32 to produce library L1. Affinity-based selections were then performed on five (H3B1, H3B2, L3B1, L3B2, H2) of

the seven libraries, whereby the concentration of target antigen was reduced over successive rounds of selection [28]. At each stage of the optimization process, scFv that were able to inhibit the binding of clone XPA067 IgG1 to the target antigen were identified. XPA.067.06 was selected as the lead candidate based on affinity, in vitro potency in functional assays, and predicted manufacturability with respect to desirable biochemical properties.

2.9. *Efficacy of reformatted antibody in vivo*

The mice were cared for in accordance with federal and local animal welfare regulations in an AAALAC-accredited facility, and the study was approved by the institutional animal care and use committee. To evaluate the in vivo efficacy of the parental and affinity-matured anti-gastrin mAbs, the effect of target neutralization was measured using gastric pH as the readout. On study day −2 (48 h before gastric fluid collection), CD-1 mice (12–14 weeks old) were treated with anti-gastrin mAbs (20 mg/kg body wt) or anti-KLH IgG1 antibody control (20 mg/kg body wt) by intraperitoneal injection. Mice were fed Napa-Nectar™ water gel instead of solid Rodent Chow. On study day −1 (24 h before gastric fluid collection), mice were fasted overnight with free access to water. On study day 0 (the day of gastric fluid collection), h-G17 (1 mg/kg body wt; Sigma, Saint Louis, MO) or PBS (pH 7.4) was injected subcutaneously. Twenty minutes after h-G17 or PBS injection, a H2R antagonist (famotidine; 30 mg/kg body wt; Sigma, Saint Louis, MO), a muscarinic M1 receptor antagonist (telenzepine; 30 mg/kg body wt; Sigma, Saint Louis, MO) or PBS was administered intravenously into each mouse. The stomach was removed 1 h later. Approximately 50 μL of gastric fluid was collected, and the pH was directly measured by using a pH meter (model D-51; Horiba Ltd., Kyoto, Japan) with microelectrode (model 9669-10D; Horiba Ltd., Kyoto, Japan).

At termination, blood samples were obtained by cardiac puncture. Blood was collected into 0.5 mL lithium heparin tubes containing 10 μL Trasylol, mixed and placed on ice. The plasma was separated by centrifugation, frozen in aliquots within 15 min of cardiac puncture and stored at −80 °C. Plasma was assayed for quantifying levels of free mAb XPA067.06.

2.10. *Measurement of XPA067.06 in mouse plasma by ELISA*

An ELISA was developed to measure XPA067.06 mAb in mouse plasma. Gastrin (Sigma, St. Louis, MO.) was reconstituted at 1 mg/mL with PBS (pH 7.4). The reconstituted gastrin solution was further diluted to 10 μg/mL in 1× PBS before use. Fifty μL of this solution was added to wells of Nunc-Immuno Maxisorp microtiter plates and antigen allowed to absorb by incubation overnight at 2–8 °C. The antigen solution was removed and 200 μL of blocking buffer (1% Bovine serum albumin (BSA) in 1× PBS containing 0.05% Tween 20) was added to all wells. Microtiter plates were then incubated for 1 h at room temperature. After blocking, the wells of each plate were washed three times with 300 μL of wash buffer (1× PBS containing 0.05% Tween 20). Standards, samples and controls were diluted in triplicate in sample diluent (1% Bovine serum albumin (BSA) in 1× PBS containing 0.05% Tween 20). XPA067.006 mAb standard solutions were prepared as serial twofold dilutions from 0.061 to 500 ng/mL. Each replicate and dilution of the standards, samples and controls (50 μL) were transferred to the blocked microtiter plates and incubated for 1 h at 37 °C. After the primary incubation, the wells were washed three times with wash buffer. Alkaline phosphatase conjugated goat anti-human IgG (gamma) antibody (Zymed Laboratories, South San Francisco, CA) was diluted 1/1000 in conjugate diluent (1% BSA in 1× PBS containing 0.05% Tween 20). Fifty microliters of the diluted conjugate was added to all wells except for the BLANK wells, which received 50 μL of conjugate diluent only. The plates were then incubated for 1 h at 37 °C. Subsequently, all wells were washed three times with wash buffer and three times with deionized water. The substrate *p*-nitrophenylphosphate (1 mg/mL in 10% diethanolamine buffer, pH 9.8) was added in a volume of 50 μL to all wells. Color development was allowed to proceed for 1 h at room temperature, after which 50 μL of 1N NaOH was added to stop the reaction.

The absorbance at 405 nm was determined for all wells using a SPECTRAmax M2 Plate Reader (Molecular Devices, Menlo Park, CA). A standard curve was plotted as A_{405} versus ng/mL of XPA067.06 standard. A regression analysis was performed and concentrations were determined for samples and controls by interpolation from the standard curve.

3. Results

3.1. *Isolating G17-specific antibodies*

More than 1000 ELISA-positive clones were identified from human antibody phage display library screening. About 500 top clones with strong ELISA signals were used for off-rate ranking analysis.

3.2. *Off-rate ranking of antibody fragments*

The screening funnel employed a high-throughput off-rate ranking method in periplasmic extracts (PPE) to allow rapid prioritization of hundreds of peptide binders identified from the phage libraries. An example of the results of analysis of one plate of ELISA positive clones is shown in Fig. 1. Clones with a ratio of specific to non-specific binding of <3, as well as those with a total binding of <20 RU were eliminated from consideration. The remaining antibody fragments were ranked according to relative dissociation rates. Sequencing of Ab fragments with off-rates $<10^{-2}$ s^{-1} were performed. By focusing on clones with the slowest off-rates and unique sequences, the number of binders was narrowed to about 65 clones for purification and interrogation in a single-point cell-based functional assays.

3.3. *Flow cytometric measurement of intracellular calcium flux to select for neutralizing antibodies to G-protein coupled receptors (GPCRs)*

Binding of gastrin to CCK-2 receptors (CCK2R) results in phospholipase C (PLC)-mediated intracellular calcium flux, a

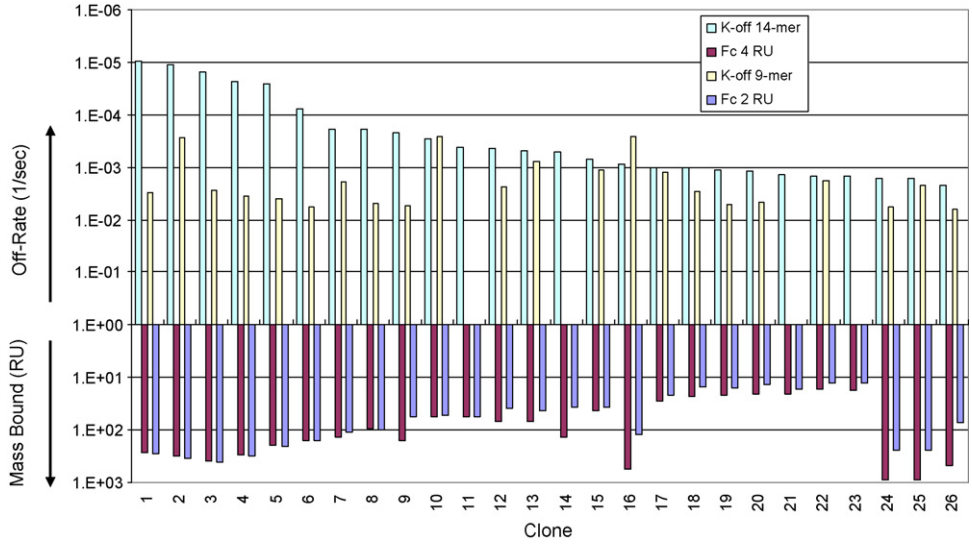

Fig. 1 – Selection of antibody fragments to move forward was aided by ranking clones by relative off-rate as well as relative mass bound. Upward pointing bar represent off-rate to two different gastrin peptides, downward pointing bars represent relative mass bound to the peptides.

portion of which results from influx of extracellular calcium through voltage-dependent L-type calcium channels. The primary functional assay for screening and characterization of target neutralizing antibodies employed flow cytometry and the fluorescent calcium indicator dyes Fluo-3 and Fura Red to measure target stimulated intracellular calcium flux in a CCK2R over-expressing cell line (AGS-TR). Because of their opposite responses to calcium binding, Fluo-3 increases in fluorescence while Fura Red decreases in fluorescence, and their different emission spectra, use of these dyes in combination results in generation of a pseudo-ratiometric signal that can reduce the influence of variables such as differences in cell size and intracellular dye concentration on the overall calcium flux response [31]. Cell loading of AGS-TR cells with Fluo-3 AM and Fura Red AM was optimized for concentration, time, and temperature to ensure the highest possible signal in response to increases in intracellular calcium concentrations.

The purified ELISA-positive anti-gastrin antibody fragments were initially screened for gastrin neutralizing activity at roughly 100-fold molar excess over gastrin (1 nM), which is typically used at the EC_{50} concentration (1 nM). Antibodies that showed over 70% neutralization of calcium flux were chosen for further characterization at a lower concentration,

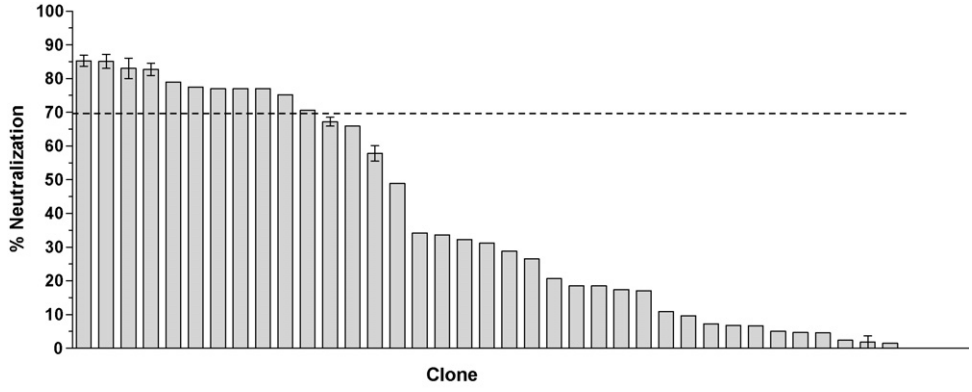

Fig. 2 – 10 nM of h-G17 (10× final concentration) was incubated with a roughly 100-fold molar excess of gastrin-binding antibody fragments for 15–30 min at room temperature. Combined gastrin-17 and antibody fragments were then diluted 1:10 by direct injection into a test tube containing CCK2R-overexpressing AGS cells (AGS-TR) loaded with the calcium sensitive dyes Fluo-3 and Fura Red. Intracellular calcium flux was monitored by flow cytometry continuously before and after sample injection. For each sample, a 10 s baseline signal was collected prior to injection. Following data acquisition, percent neutralization for each clone was calculated using gastrin-17 and unstimulated controls. Clones were then ranked according to percent neutralization. Each bar represents a single antibody fragment clone. The horizontal dashed line represents the cut-off threshold of the 70% neutralization.

Table 1 – Affinity measurements and calcium flux dose response data of the two top anti-gastrin antibodies from human phage libraries

Antibody	K_a (1/s)	K_d (M^{-1})	K_D (nM)	Ca^{2+} Flux IC_{50} (nM)
XPA067	2.3e5	9.2e−4	5	0.76 ± 0.38
XPA061	1.6e5	1.6e−3	9.9	1.34 ± 0.21

e.g., 2× molar excess, to further differentiate the neutralization potencies of the antibodies. Eleven top clones that showed a high degree of neutralization at lower concentrations (Fig. 2) were reformatted to whole IgG. Similar ranking was obtained from screening the scFv and Fab fragments in an ERK phosphorylation assay (data not shown).

3.4. Analysis by neutralization and affinity assays of reformatted IgG antibodies

Anti-gastrin IgG was isolated from cell supernatants and analyzed by calcium flux neutralization assay as described previously. Thirteen reformatted whole IgG anti-gastrin candidates antibodies were tested at IC_{25} (0.3 nM) and IC_{75} (1.7 nM). XPA067 was also tested at 67 nM as a control for maximum neutralization in the experiment. Analysis of reformatted antibodies showed that XPA067 at 67 nM exhibited approximately 85% neutralization at 1.7 nM, approximately 55% calcium flux neutralization, and at 0.3 nM, all 13 candidate antibodies demonstrated 10% or less neutralization. Dose–response studies indicate the IC_{50}s of XPA067 and XPA061 are 0.76 ± 0.38 and 1.34 ± 0.21, respectively (Fig. 3 and Table 1). In addition, the full affinity values of the reformatted IgGs were estimated by BIACORE® (Table 1). The top candidate, XPA067, was selected for affinity maturation based on two criteria: (1) calcium flux IC_{50} value and (2) K_D measurement.

3.5. Gastrin PK/PD model and output

The fraction of receptor bound to gastrin needed to prevent tumor growth is not known. However, the vaccination process against gastrin in patients, called Gastrimmune™, has been described in the literature [25,32]. Ranges of affinity cited for Gastrimmune™ are 0.16–0.41 nM, and the titer has been cited as 50 to 500-fold in excess of gastrin. As the maximal concentration of gastrin is 0.15 ng/mL, and assuming 1:1 molar stoichiometry, then maximum conditions of Gastrimmune™ levels plus affinity could be estimated. These Gastrimmune™ values were simulated using the PK/PD model under steady state conditions, and the simulated fraction receptor bound by gastrin was determined to be 0.0156. This fraction receptor bound by gastrin served as the goal to achieve for the dosing regimen of the mAb. Using the PK/PD model, for an antibody with 5 nM affinity for gastrin, 4 mg/kg weekly doses were required to maintain fractional receptor bound to gastrin levels of 0.0156 or below, as shown in Fig. 4B. Also plotted are the free gastrin levels; and the fraction of receptor bound to gastrin with time. The result did not support a compelling dosing regimen nor commercially attractive product profile, thereby driving the decision to affinity mature the lead candidate, XPA067, for improved potency.

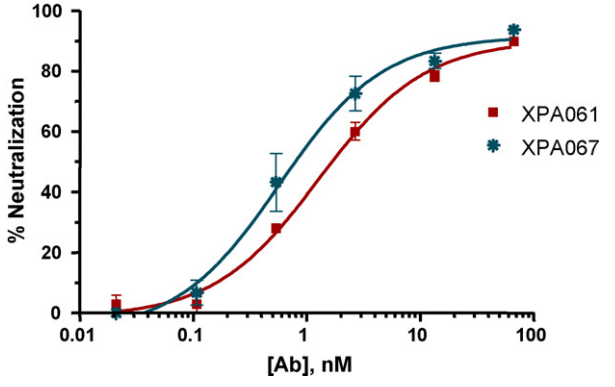

Fig. 3 – The two top clones from the screening campaign, XPA061 and XPA067, were reformatted and expressed as human IgG1 antibodies and tested in the flow cytometry-based calcium flux assay in a 6-point concentration response experiment. The shown data represents 2 (for XPA061) and 3 (for XPA067) repetitions of the experiment.

3.6. Affinity maturation of anti-gastrin XPA067

The affinity of an antibody can be improved via mutagenesis of individual amino acids in the variable region [33–35]. In this study, the improvement in affinity was achieved by random mutagenesis of CDRs in combinatorial antibody phage display libraries. Libraries of mutants were selected by phage display and screened by competition with the parent antibody for gastrin binding. Clones with the highest affinities for gastrin were chosen for further analysis.

The affinity-matured antibodies showed improved affinities compared to the parent antibody, XPA067 (Fig. 5A). These results demonstrated that the affinity maturation process successfully generated antibodies with higher affinity than the parent antibody. The top clones with greatest improved affinity were selected for reformatting to full IgG. The sensorgrams for the top two affinity matured XPA067.18 and XPA067.06 IgGs are shown in Fig. 5B.

3.7. Efficacy of affinity matured antibodies in vitro

To further rank the affinity matured antibodies in terms of the efficacy of gastrin neutralization, an in vitro calcium flux assay was performed. The results indicated that the reformatted antibodies XPA067.18 and XPA067.06 had the highest capacity to neutralize gastrin activation of calcium flux in AGS-TR cells (Fig. 6A), which correlated with the affinity ranking data. Subsequently, a full dose–response calcium flux assay using the parent mAb, XPA067, and the affinity matured mAbs, XPA067.06 and XPA067.18, was carried out. Parent antibody XPA067 demonstrated approximately 50% gastrin neutralization at a concentration of approximately 1 nM whereas XPA067.06 reached 50% neutralization at about 0.17 nM and XPA067.18 showed 50% neutralization at about 0.2 nM (Fig. 6B). These data demonstrate that the affinity-matured antibodies also have a higher potency for gastrin neutralization than the parent antibody.

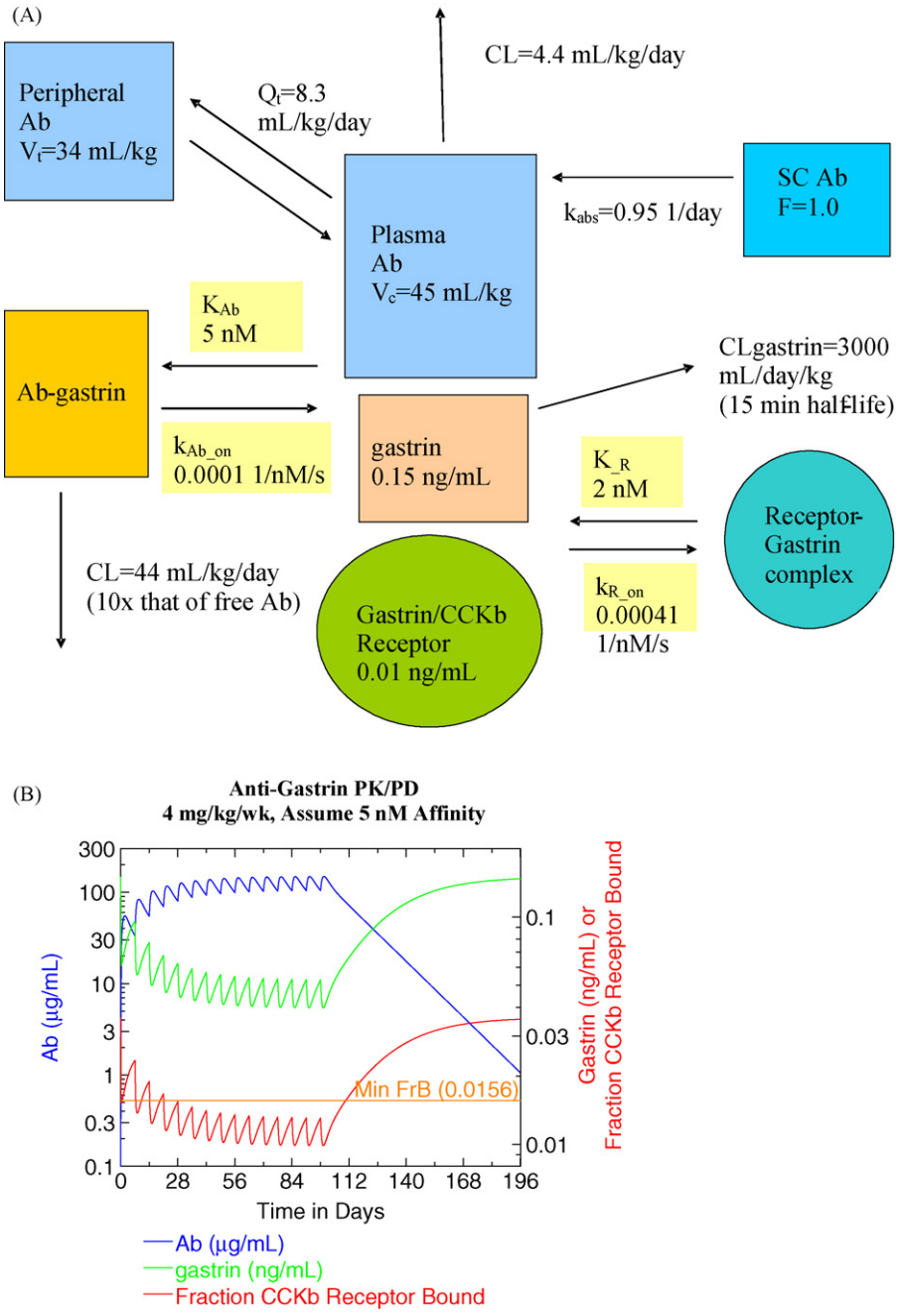

Fig. 4 – (A) The PK/PD was developed based on known pharmacokinetics of human antibodies in humans without target, literature values of maximal gastrin levels, the half-life of gastrin, and the affinity of gastrin for its natural receptor, CCK-B, on mucosal/cancer cells. (B) Shown are the projected concentration–time profiles for mAb administered subcutaneously at 4 mg/(kg week) with an affinity for gastrin of 5 nM, the projected free serum gastrin levels, and projected fraction of receptor bound to gastrin, based on the PK/PD model described in (A).

3.8. *Sequence analysis for selecting the lead mAb*

The affinity and in vitro potency of XPA067.06 and XPA067.18 are very similar. In order to select the ideal lead candidate, the variable region sequences of the two antibodies were analyzed to predict each clone's physical attributes for better stability, such as solubility, optimal pH and presence or absence or potentially labile degradation sites. The variable regions were then examined for sites particularly prone to degradation mechanisms that might affect binding or activity, with attention focused on CDR's. Finally, the lead antibody, XPA067.06, was selected based on a weighted analysis comparing all of the factors described above.

3.9. *Efficacy of lead antibody in vivo*

Monitoring pH changes in gastric fluid can serve as a pharmacological end point to determine the effect of a drug

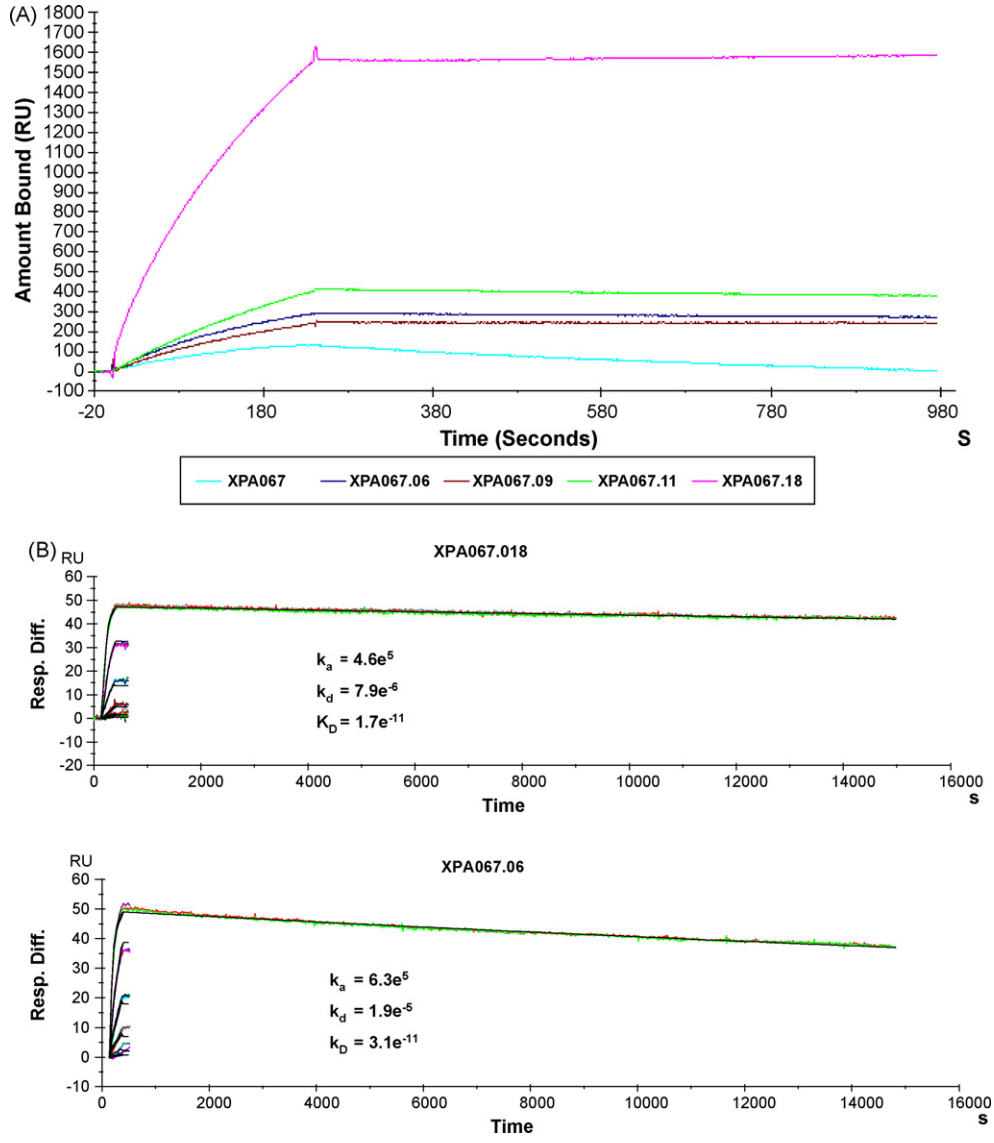

Fig. 5 – (A) Single concentration kinetic ranking of four affinity matured anti-gastrin scFV-Fc and the parental XPA067 mAb. (B) Sensorgram of full kinetic analysis of two affinity matured IgGs. Each antibody tested at six concentrations in triplicate and fit globally to a 1:1 langmuir model. Top XPA067.18; bottom XPA067.06.

targeting gastrin activity. To this end, two complementary models were established using famotidine and telezepine. Although the pathways of gastric acid secretion stimulated by gastrin (acid simulator; signal through CCK-B receptor), famotidine (acid antagonist; signal through H2 receptor) and telenzepine (acid antagonist; signal through M1 receptor) are different; we hypothesize that the pH values of gastric fluid can be compensated by the optimized doses and administration schedules with the combination of the simulators and antagonists of gastric acid.

Studies employing the gastric pH models indicate that antagonists like famotidine and telenzepine can increase the gastric pH in out-bred CD-1 mice (Fig. 7). Thus, neutralizing anti-gastrin antibody (as seen with other antagonists) should block the function of endogenous gastrin thereby increasing the gastric pH levels. However, none of the candidate anti-gastrin antibodies showed cross-reactivity with mouse or rat gastrin (data not shown). To overcome this obstacle, the models were modified to include exogenous administration of h-G17 to CD-1 mice. Our data indicated human gastrin, h-G17, can interact with mouse gastrin receptors and mediate gastric acid secretion (Fig. 7). The inhibition of gastric acid secretion induced by famotidine and telenzepine can be neutralized by administration of exogenous h-G17, apparently functioning through mouse receptors (Fig. 7). Therefore, sophisticated mouse gastric pH models were developed based on the optimized doses and administration schedules of combining famotidine/telenzepine and h-G17 (Fig. 8). By testing the anti-gastrin mAb in these optimized gastric pH models, the in vivo neutraliza-

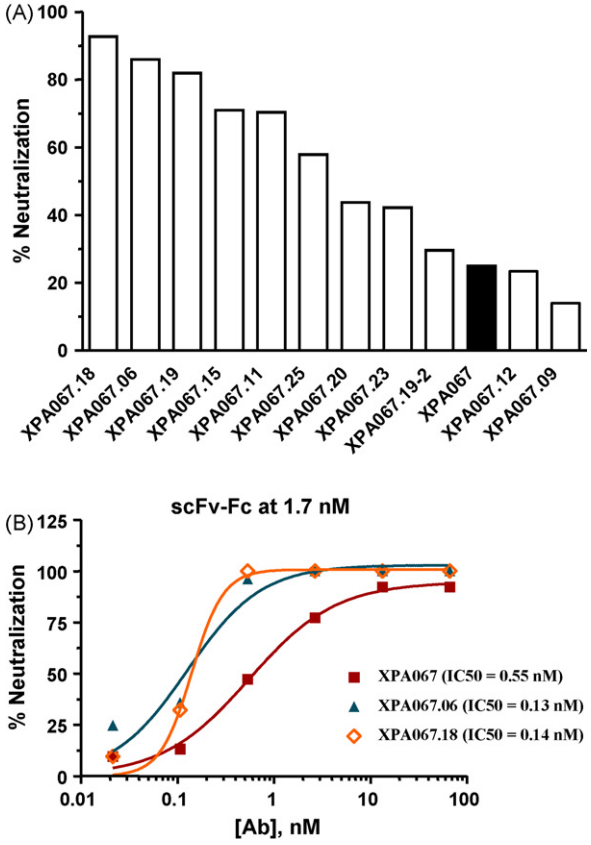

Fig. 6 – (A) To aid in selection of an affinity matured lead candidate, affinity matured scFv-Fc clones were tested for neutralization of recombinant h-G17 at the previously determined reference antibody IC_{75}. (B) The two top affinity-matured clones, XPA067.06 and XPA067.18, were reformatted and expressed as human IgG1 antibodies and tested in a 6-point concentration response experiment ($n = 3$).

tion activity of h-G17 could be measured by the readout of gastric pH (Fig. 8).

These data indicated that administration of famotidine alone neutralized endogenous mouse gastric acid and raised the pH levels in the stomach to approximately pH 5.5, whereas administration of exogenous h-G17 and famotidine reduced the stomach pH levels to approximately pH 2 (Fig. 9). Thus, under the certain doses, famotidine could not neutralize the effect of exogenous gastrin. Administration of the parental anti-gastrin antibody XPA067, h-G17, and famotidine demonstrated a mild increase in stomach pH, to pH 2.5 (Fig. 9). However, administration of the affinity matured anti-gastrin antibody XPA067.06 further increased the pH in the stomach to approximately pH 4 (Fig. 9). Similarly, administration of telenzepine alone neutralized endogenous mouse gastric acid and raised the pH levels in the stomach to approximately pH 3, while administration of exogenous h-G17 and telenzepine reduced the stomach pH levels to approximately pH 1.3 (Fig. 10). Administration of the parental anti-gastrin antibody XPA067, h-G17, and telenzepine demonstrated a mild increase in stomach pH, to pH 1.5 (Fig. 10). Again, the affinity matured anti-gastrin antibody XPA067.06 further increased the pH in the stomach to approximately pH 2 (Fig. 10). To compare the famotidine- and telenzepine-gastric pH models, the famotidine model gave a broader range of pH values with larger variations than the telenzepine model did (Figs. 9 and 10), although both models demonstrated the same trends in responding to the administrations of the respective anti-gastrin mAbs.

Taken together, these data demonstrate that normal levels of gastric acid secretion are decreased by administration of the selective H2R antagonist, famotidine, or the muscarinic M1 receptor antagonist, telenzepine. In addition, these effects can be abolished by pre-introducing exogenous h-G17 to CD-1 mice. Elevation of gastric pH was observed after administration of the neutralizing anti-gastrin antibodies. These results show that the anti-gastrin mAbs

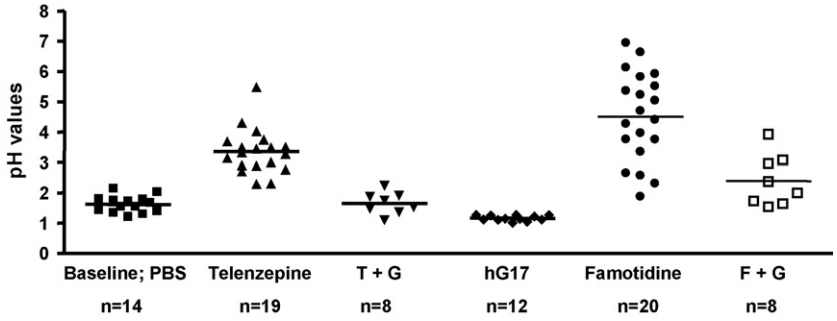

Fig. 7 – Basal- and pharmacological stimulated- and inhibited-gastric pH levels in CD-1 mice. Mice 12–14 weeks old were given Napa-Nectar gel food two days before the treatments and fasted overnight with free access to water the day before treatments. 200 μL PBS (baseline), a muscarinic M1 receptor antagonist (telenzepine "T"; 30 mg/kg body wt) or a H2R antagonist (famotidine "F"; 30 mg/kg body wt) was administered intravenously. Three hundred microliters of h-G17 ("G"; 1 mg/kg body wt) was administered subcutaneously. T + G and F + G represent the combination treatments of h-G17 plus telenzepine and h-G17 plus Famotidine, respectively. Animal number (*n*) for each treatment group is listed on the bottom of the figure. Each symbol (filled squares, baseline; filled triangles, telenzepine; filled inverted triangles, T + G; filled diamonds, h-G17; filled circles, famotidine; open squares) represents the pH value of the data point corresponding to an individual animal. Horizon bars represent mean values.

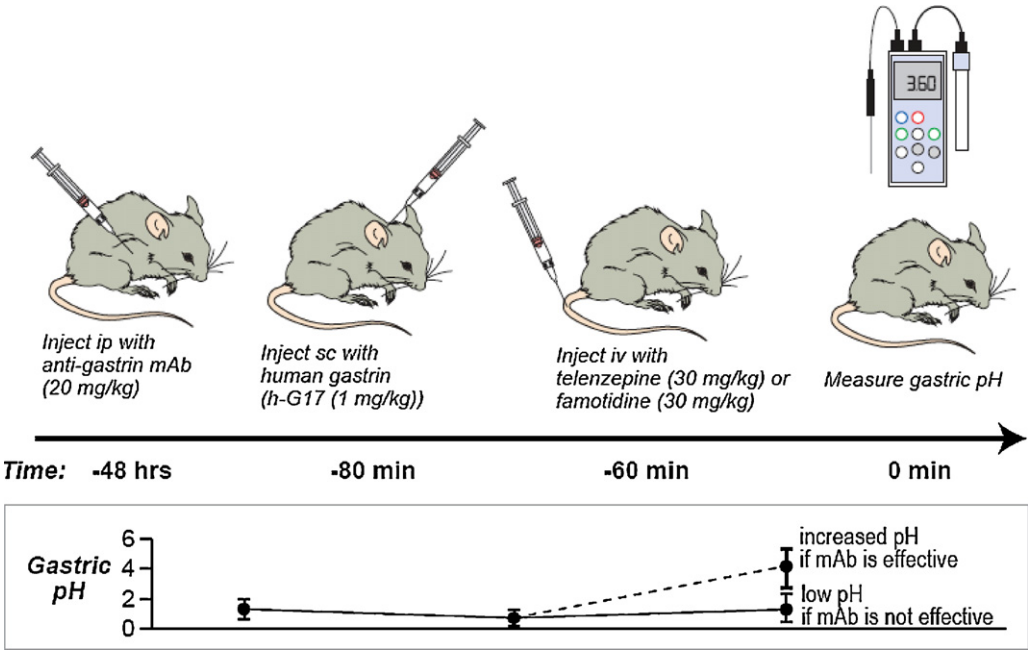

Fig. 8 – Diagram of the gastric pH models for testing the efficacy of the anti-gastrin antibodies. The baseline of gastric pH in CD-1 mouse is ~pH 1.5. Since the human anti-gastrin mAbs do not cross-react with murine gastrin, administration of the mAb does not alter mouse stomach pH. and the pH values will remain low after mAb administration. However, h-G17 can interact with mouse CCK-2 receptors and stimulate the gastric acid secretion, so exogenous h-G17 can further acidify the stomach to ~pH 1. If a human anti-gastrin mAb can neutralize the exogenous h-G17, the subsequent administration of the gastrin antagonists telenzepine or famotidine will raise the gastric pH. Conversely, if an anti-gastrin mAb does not neutralize the exogenous h-G17, the effect of the gastrin antagonists will be diminished due to the excess of h-G17 and the gastric pH will remain low.

neutralize exogenous h-G17 in vivo. These data further indicate that the affinity-matured antibody, XPA067.06, is a more potent gastrin-neutralizing agent than the parent antibody, XPA067, which is consistent with the in vitro results and PK/PD model predictions.

3.10. *Levels of fasting plasma mAb after the completion of the administration schedule with h-G17 and XPA067.06*

The in vivo efficacy of XPA067.06 was further confirmed by measuring the free antibody remaining in circulating plasma

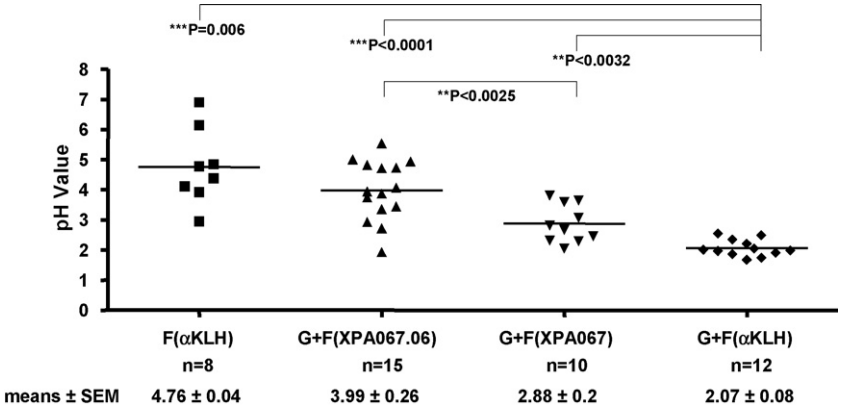

Fig. 9 – In vivo efficacy of the anti-gastrin mAbs in the famotidine gastric pH model. The animal number (*n*) for each treatment group is at the bottom of the figure. Each symbol (filled squares, αKLH antibody + famotidine; filled triangles, XPA067.06 antibody + h-G17 + famotidine; filled inverted triangles, XPA067 antibody + h-G17 + famotidine; filled diamonds, αKLH antibody + h-G17 + famotidine) represents the pH value of the data point corresponding to an individual animal. Data are presented as means ± S.E.M. Statistical differences were evaluated using the unpaired *t*-test with Welch's correction and the *p*-values are listed at the top of the figure.

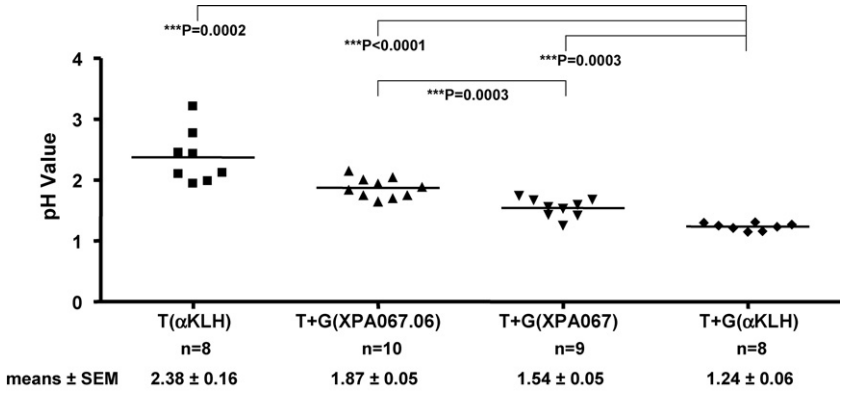

Fig. 10 – In vivo efficacy of the anti-gastrin mAbs in telenzepine gastric pH model. The animal number (*n*) for each treatment group is listed at the bottom of the figure. Each symbol (filled squares, αKLH antibody + telenzepine; filled triangles, XPA067.06 antibody + h-G17 + telenzepine; filled inverted triangles, XPA067 antibody + h-G17 + telenzepine; filled diamonds, αKLH antibody + h-G17 + telenzepine) represents the pH value of the data point corresponding to an individual animal. Data are presented as means ± S.E.M. Statistical differences were evaluated using the unpaired *t*-test with Welch's correction and the *p*-values are listed at the top of the figure.

after target neutralization. At 48 h before plasma collection, CD-1 mice (12–14 weeks old; $n = 8$ per group) were treated with anti-gastrin mAbs (20 mg/kg body wt) or anti-KLH IgG1 antibody control (20 mg/kg body wt) by intraperitoneal injection. Based on our PK/PD modeling, antibody levels will reach β phase in 48 h (data not shown). At 24 h before h-G17 challenge, mice were fasted overnight with free access to water. At 20 min before plasma collection, mice were treated with h-G17 (1 mg/kg body wt) or PBS (pH 7.4).

A h-G17 specific ELISA was developed to measure XPA067.06 mAb in mouse plasma (see detail in Section 2).

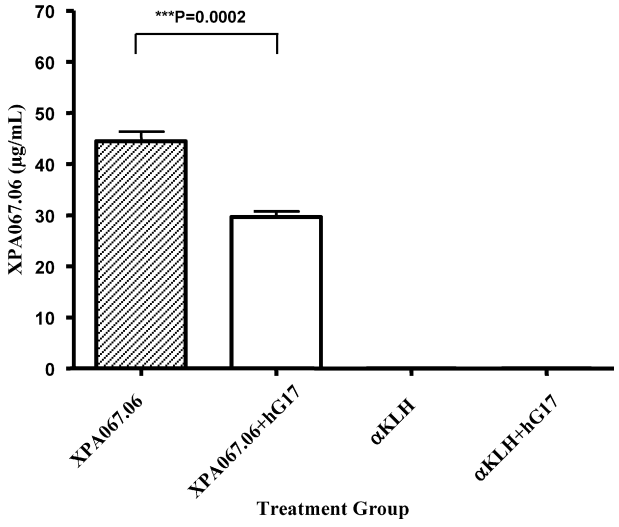

Fig. 11 – XPA067.06 levels in the plasma of the XPA067.06 and/or h-G17 treated CD-1 mice. Average XPA067.06 concentrations (μg/mL) with standard error bars are shown in mice treated with XPA067.06 only (hatched bar), XPA067.06 with h-G17 (open bar), anti-KLH only, and anti-KLH with h-G17. Statistical differences were evaluated using the Mann–Whitney test (nonparametric t-test) and *p*-value is at the top of the figure.

Fig. 11 shows XPA067.06 concentrations with and without the administration of h-G17. The data indicates that in vivo neutralization effect was achieved. The average concentrations of XPA067.06 mAb are 44.5 μg/mL and 29.7 μg/mL in the groups with and without h-G17 treatment, respectively. The hG-17 treated group had free antibody approximately 1.5 fold lower than the untreated group.

4. Discussion

Gastrin has been implicated as a growth factor in a variety of cancer types, such as gastric, pancreatic and colorectal carcinoma [9–11]. In addition to oncology indications several GI disorders also are associated with gastric acid secretion, including gastric and duodenal ulcers, autoimmune gastritis, atrophic body gastritis [36], inflammatory bowel disease [37], and Zollinger–Ellison syndrome [38]. In order to provide an effective and long-lasting, targeted therapy, a high affinity fully h-G17 neutralizing mAb XPA067.06 was developed.

To predict the antibody characteristics necessary to help guide development decisions, an in silico PK/PD model was developed. The model took into account the half-life of gastrin, a weighted average of post-parandial and fasting serum levels of gastrin in the human body (~50 pM for 2 kDa MW) [25], the affinity of gastrin for its cognate receptor, CCK-2, on mucosal/cancer cells, and the expected PK profiles for an antibody. Once established, various dosing regimen were simulated to assess the clinical and commercial feasibility of the therapeutic candidate. Based on the outputs it was determined that sub-nM affinity would be required to achieve the target product profile and the lead anti-gastrin 5 nM neutralizing antibody, XPA067, was affinity maturated over 150-fold to the 30 pM XPA067.06 product candidate.

Indeed, preclinical characterization studies demonstrated the parental 5 nM XPA067 antibody, neutralized 90% of the gastrin-induced calcium flux activity in vitro at a

concentration of about 10 nM and had modest in vivo neutralizing activity in vivo in two animal models. However, the 30 pM affinity matured antibody, blocked 90% of the gastrin-mediated activity in vitro at a concentration of about 0.2 nM. Importantly, XPA067.06 produced marked neutralization of gastrin's acid-stimulating effects in vivo in the same animal models. Taken together these data also demonstrate the utility of the in silico modeling to guide therapeutic candidate development decisions for creating a molecule that possesses biologically meaningful activity in vivo. In summary, these studies demonstrate that XPA067.06 significantly inhibits h-G17 stimulated acid output in vitro and in vivo and has potential to clinically impact diseases associated with gastrin-stimulated gastric acid secretion.

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