

Mechanistic Investigations of Diarrhea Toxicity Induced by Anti-HER2/3 Combination Therapy

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Abstract

Combination of targeted therapies is expected to provide superior efficacy in the treatment of cancer either by enhanced antitumor activity or by preventing or delaying the development of resistance. Common challenges in developing combination therapies include the potential of additive and aggravated toxicities associated with pharmacologically related adverse effects. We have recently reported that combination of anti-HER2 and anti-HER3 antibodies, pertuzumab and lumretuzumab, along with paclitaxel chemotherapy in metastatic breast cancer, resulted in a high incidence of diarrhea that ultimately limited further clinical development of this combination. Here, we further dissected the diarrhea profile of the various patient dose cohorts and carried out *in vitro* investigations in human colon cell lines and explants to decipher the

contribution and the mechanism of anti-HER2/3 therapeutic antibodies to intestinal epithelium malfunction. Our clinical investigations in patients revealed that while dose reduction of lumretuzumab, omission of pertuzumab loading dose, and introduction of a prophylactic antidiarrheal treatment reduced most severe adverse events, patients still suffered from persistent diarrhea during the treatment. Our *in vitro* investigations showed that pertuzumab and lumretuzumab combination treatment resulted in upregulation of chloride channel activity without indication of intestinal barrier disruption. Overall, our findings provide a mechanistic rationale to explore alternative of conventional antidiarrheal medication targeting chloride channel activity to mitigate diarrhea of HER combination therapies. *Mol Cancer Ther*; 17(7); 1464–74. ©2018 AACR.

Introduction

Targeted cancer therapies can cause diarrhea by various pathophysiologic mechanisms (1), and careful evaluation is required to determine the most effective antidiarrheal treatment for individual patients. Ideally, a systematic evaluation of the mechanism mediating diarrhea and the contribution of the pharmacologic and nonpharmacologic effects of the therapies is necessary. The clinical investigation includes the analysis of duration, severity, constellation of signs and symptoms of the toxicity, and if possible the histopathology analysis of damaged tissue. The limited number of patients exposed to the experimental therapies, particularly at the early phase of develop-

ment, the incomplete evaluation of the contribution of each individual drug when given in combination, and the lack of supportive biopsies often lead to incomplete conclusion. *In vitro* investigations using cellular models of the gastrointestinal mucosa are alternative investigational tools that can successfully provide molecular and cellular insights into the safety profile of drug candidates (2, 3) and guide the implementation of development strategies and mitigation plans. Here, an *in vitro* approach has been applied to elucidate the mechanisms underlying the high incidence of chronic diarrhea recently reported in a phase Ib study evaluating the safety and clinical activity of a targeted combination therapy consisting of the investigational anti-HER3 drug lumretuzumab and the approved anti-HER2 drug pertuzumab, in patients with HER3-positive, HER2-low metastatic breast cancer also receiving the chemotherapeutic agent paclitaxel (4).

Lumretuzumab is a glyco-engineered monoclonal antibody (mAb) selectively binding to the extracellular subdomain I of HER3 and was investigated for the treatment of patients with HER3-expressing solid tumors (5). In the first-in-human study, lumretuzumab monotherapy showed a favorable safety profile across all dose groups of patients, up to 2,000 mg being the highest dose tested. Downregulation of HER3 receptor activity in lumretuzumab-treated patient tumor biopsies along with linear serum pharmacokinetics, suggesting receptor saturation, indicated optimal biological activity of lumretuzumab at doses at and above 400 mg per patient (6). Lumretuzumab was also evaluated in combination with anti-HER1 therapies (cetuximab or erlotinib), showing a manageable safety profile, although

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diarrhea appeared to be increased in frequency for higher lumretuzumab doses (7).

The inhibition of HER signaling by displacing the HER3 ligand (8) is expected to have a strong antitumor activity, particularly in combination with anti-HER1 and anti-HER2 therapies, and to block an important escape mechanism observed with anti-HER1 or anti-HER2 therapies (9). The proposed combination of lumretuzumab and pertuzumab, aimed at achieving a complete inhibition of the HER heterodimers (HER2:HER3, HER2:HER1, and HER1:HER3), was associated with an unexpected high incidence of grade 3 diarrhea despite mitigation efforts by dose modifications of lumretuzumab, pertuzumab, and/or paclitaxel and prophylactic loperamide treatment. The chronic diarrhea remained the major side effect of the combination therapy and the therapeutic window was too narrow to warrant further clinical development (4).

While diarrhea is a known side effect associated with paclitaxel and pertuzumab treatment in solid tumor cancer patients (1), the severity, early onset, and duration of this gastrointestinal toxicity in the triple combination with lumretuzumab were not expected based on the known safety profile of the individual therapeutic agents. Thus, diarrhea was reported in less than 50% of patients treated with paclitaxel and only 1% of patients had grade ≥ 3 diarrhea (6, 10). Pertuzumab used as single agent or in combination with an additional anti-HER2 mAb trastuzumab and the taxane agent docetaxel, revealed less than 10% of grade ≥ 3 diarrhea with an overall (all grade) incidence rate of 60% (11–13). Lumretuzumab as monotherapy was considered well tolerated in patients, with no dose limiting toxicities reported up to a 2,000 mg dose. The overall incidence of diarrhea was 46.8% (all doses, all grades) with no discernible difference between dose cohorts to the exception of grade ≥ 3 diarrhea, which was reported only for patients from the extension cohort at 2,000 mg (5).

Based on the clinical safety profile of the diarrhea reported in the triple combination, we speculated that the comprehensive inhibition of HER family signaling pathways may account for the additive gastrointestinal complications in patients (8, 14). Indeed, it has been hypothesized that blockage of HER signaling may (i) directly damage the intestinal mucosa and impair healing of the intestinal epithelial barrier, leading to reduced absorption of water and electrolytes, and/or (ii) induce secretory diarrhea by direct modulation of chloride channels (15–17). These mechanisms, however, remain only hypothetical because clinical findings of diarrhea in the context of targeted therapies have not been tested experimentally and few histopathologic analyses are available.

Here, to provide insights into the mechanisms underlying the high incidence of diarrhea in the patients treated with anti-HER2 and anti-HER3 combination therapy (4), we took a closer look at the diarrhea profile in relation to the dose regimen, duration, contribution of individual therapy, and prophylactic use of anti-diarrheal treatment. In addition, we experimentally verified the effects of pertuzumab and lumretuzumab on colon epithelial cell proliferation and survival, intestinal barrier integrity, and chloride channel activity *in vitro* and *ex vivo*. Altogether, our analyses suggest that the aggravated diarrhea observed after concurrent inhibition of the HER2 and HER3 receptors results from an upregulation of chloride channel activity without tissue damage.

Materials and Methods

Lumretuzumab and pertuzumab

Lumretuzumab and pertuzumab antibodies were described previously (11, 18).

Clinical study design

The study NCT01918254 was an open-label, nonrandomized, dose escalation, multicenter phase Ib study in patients with metastatic breast cancer expressing HER3 and HER2 proteins, investigating the safety, PK, pharmacodynamics (PD), and clinical activity of lumretuzumab in combination with pertuzumab and paclitaxel in patients with HER3-positive, HER2-low metastatic breast cancer. The study was conducted in two parts: a dose escalation with up to 6 eligible patients per cohort, followed by an extension part. Patients continued treatment until disease progression, unacceptable toxicity, or consent withdrawal (4).

Study approval and ethics

Local ethics committee approval was obtained, and all patients provided written informed consent. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki in nine centers in Denmark, France, Germany, and Spain.

Study treatment

For the study NCT01482377 (monotherapy of lumretuzumab), see details in Meulendijks and colleagues (6).

In the study NCT01918254, patients received intravenously lumretuzumab every 3 weeks (q3w) at 1,000 mg dose ($N = 2$ for cohort 1) or 500 mg ($N = 20$ for cohort 2 and $N = 13$ for cohort 3) in combination with pertuzumab and paclitaxel. Pertuzumab was administered intravenously in cohorts 1 and 2 on day 1 of cycle 1, at a loading dose of 840 mg followed by 420 mg on day 1 of each subsequent cycle. Patients in cohort 3 started at a reduced dose of pertuzumab, i.e., 420 mg for cycle 1 and all subsequent cycles. Paclitaxel was administered intravenously weekly, i.e., on day 1, 8, and 15 of each 3-weekly treatment cycle (q3w), at a dose of 80 mg/m².

Safety assessments

Safety assessments consisted of monitoring and recording adverse events (AE), including serious adverse events (SAE), measurement of protocol-specified safety laboratory assessments (hematology, biochemistry, and urinalysis), measurement of protocol-specified vital signs (heart rate and blood pressure), and electrocardiogram. Severity of an AE was assessed using the predefined grading criteria from the Common Terminology Criteria for Adverse Events, version 4.03 (CTCAEv4.03).

Clinical management and risk mitigation of diarrhea

In the study NCT01918254, the management of diarrhea included a dose delay, reduction of paclitaxel as recommended in the corresponding labels. Lumretuzumab and pertuzumab doses could be interrupted or delayed to manage AEs, but dose reductions were not permitted. In case of grade 3 diarrhea, dosing of paclitaxel/pertuzumab/lumretuzumab would be delayed immediately. If diarrhea grade ≥ 2 was associated with impaired quality of life for > 21 days, the study treatment would be stopped permanently. If diarrhea resolved to grade ≤ 1 , treatment was to be restarted with paclitaxel (at a reduced dose of 60 mg/m²)/pertuzumab/lumretuzumab, and in cohort 3, pertuzumab was

to be discontinued (based on clinical judgment). If there was a reoccurrence of grade 3 diarrhea, then pertuzumab was to be discontinued (if not already done at first occurrence) or pertuzumab and lumretuzumab permanently.

In cohort 3, loperamide was introduced at the initiation of pertuzumab treatment, starting at 4 mg prior to the first dose followed by 2 mg every 4 hours until day 4, then 2 mg every 6 hours until the end of the first cycle (i.e., 3 weeks) and at subsequent cycles as clinically indicated (19).

Statistical analysis of clinical data

All patients who received at least one dose of study medication were included in the safety population (for details see ref. 4) and considered here for the analyses on diarrhea. We used the Kaplan–Meier method to estimate median time to a diarrhea event and to draw the respective curves with 95% confidence intervals. In order to also provide a comparison with monotherapy data, we included the cohort of patients treated with 2,000 mg lumretuzumab monotherapy from the study NCT01482377, which was a phase Ia open-label, non-randomized, dose-escalating, multicenter study investigating the safety, PK, PD, and clinical activity of single-agent lumretuzumab in patients with metastatic or advanced HER3-positive carcinomas (for details see ref. 6).

Gene expression analysis of HER2 and HER3 in cell models

Gene expression analysis was performed based on the human normal colorectal sample set generated by the TCGA Research Network (<http://cancergenome.nih.gov/>) as well as five selected colon cell lines from the Roche pRED CELLO cell bank (Roche Diagnostics GmbH). Gene expression was profiled using in-house tools, and reads per kilobase per million reads (RPKM) were computed as previously described using in-house software (20).

Caco2 culture

Caco2 cells (ATCC, HTB-37) were cultivated according to the manufacturer's instructions in Caco2 medium [DMEM/F12-Glutamax, nonessential amino acid (1%), penicillin/streptomycin (1%) (Gibco, 31331-028) (Gibco, 11140-035) (Gibco: 15140-122), 20% FBS (Gibco, 16000-044)] for two passages, then cultivated in Caco2 medium containing 10% FBS for up to 50 passages. No mycoplasma testing was performed during the duration of the study.

Immunoblot analysis

Caco2 cells were plated at 500,000 cells/well in 6-well plates in Caco2 medium and grown until confluence. Cells were exposed to saline or mAb at concentrations ranging from 1 to 300 µg/mL (covering the estimated intestinal concentrations at clinical doses used in the current study, i.e., 20–30 µg/mL; ref. 6) for 60 minutes, followed by exposure to 5 ng/mL of heregulin (Sigma-Aldrich, SRP3055) for 10 minutes. Cells were washed with cold PBS and lysed in 100 µL of ice cold RIPA buffer [60 mmol/L Tris-HCl at pH 7.4, 150 mmol/L NaCl, 0.25% SDS, 1% NP40, 1× PhosSTOP (04906845001, Roche Diagnostics) and 1× complete protease inhibitor (Roche, 04693124001)]. Protein content in cell lysis was measured by BCA Kit (Thermo Fisher, 23225), and 20 µg of total proteins was separated by electrophoresis using a 5% to 20% polyacrylamide gel. Immunoblot analyses were carried out using the

following antibodies: phospho-HER2-Tyr1196 (Cell Signaling Technology, 6942S), HER2 (Cell Signaling Technology, 2165S), phospho-HER3-Tyr1197 (Cell Signaling Technology, 4561S), HER3 (Cell Signaling Technology, 4754S), phospho-Akt-Ser473 (Cell Signaling Technology, 4060), Akt (Cell Signaling Technology, 4691), phospho-Erk1/2-Thr202/Tyr204 (Cell Signaling Technology, 4370), Erk1/2 (Cell Signaling Technology, 9102), phospho-EGFR-Tyr1173 (Novus Biologicals, NBP110-56948), EGFR (Novus Biologicals, NBP1-61853), and GAPDH (Sigma-Aldrich, G8795). The CHEMIDOC MP Imaging System (Bio-Rad) was used to scan the membranes.

Cell proliferation and cytotoxicity

For proliferation and cytotoxicity assessment, Caco2 were seeded into 96-well plates (353219, Corning) at a density of 10,000 cells/well in Caco2 medium and grown for 48 hours prior to treatment with mAbs. mAbs were added to the cell culture at concentrations ranging from 0.8 to 300 µg/mL in a final volume of 100 µL of Caco2 medium containing 10% FBS and 5 ng/mL of heregulin. Saline served as vehicle control. Medium containing mAbs and heregulin was refreshed after 3 days. After 5 days of mAb exposure, cell viability was determined by measurement of intracellular ATP levels using the CellTiter-Glo Luminescent Cell Viability Assay (G7571, Promega) according to the manufacturer's instructions. For cytotoxicity, supernatants were analyzed for the presence of a dead cell protease using the CytoTox-Glo Cytotoxicity Assay (Promega, G9290) according to the manufacturer's instructions. As positive control of drug-induced cytotoxicity, Caco2 cells were treated with 0.1, 1, and 10 µmol/L of staurosporine (Sigma-Aldrich, S4400) for 24 hours. DMSO served as vehicle control. Mean concentrations and standard deviations of three biological replicates were calculated and normalized on vehicle control.

Intestinal barrier integrity assay

For intestinal barrier integrity testing, Caco2 cells were grown in three-lane OrganoPlates (Mimetas BV) as described before (21). Briefly, 2 µL of 4 mg/mL Collagen I gel (AMSbio Cultrex 3D Collagen I Rat Tail, 5 mg/mL, cat. 3447-020-01), 100 mmol/L HEPES (Life Technologies, 15630-122), and 3.7 mg/mL NaHCO₃ (Sigma, cat. S5761) were dispensed in the gel inlet and incubated 30 to 45 minutes at 37°C. Caco2 cells were seeded in the medium channel at a density of 20,000 cells per channel and grown until confluent, as described before. After 5 to 7 days of culture in the OrganoPlate, Caco2 formed tight confluent tubes allowing for drug testing. Medium was changed for Caco2 medium containing 5 ng/mL of heregulin along with up to 300 µg/mL of pertuzumab and lumretuzumab or up to 200 nmol/L of paclitaxel (Sigma-Aldrich, T7191; ref. 22) as indicated in the figure legends. Saline and DMSO served as negative controls, respectively. After 48 hours of treatment, medium in the apical (luminal) perfusion channel was replaced by medium containing 0.125 mg/mL FITC-dextran (150 kDa, Sigma # 46946). Leakage of the fluorescent probe from the lumen of the tubular structure into the ECM compartment was automatically imaged using the Operetta high content imager (PerkinElmer) at 4× magnification and analyzed using Harmony software (PerkinElmer). The image analysis script relies on the bright field channels to localize the three tubes on each well as illustrated in Supplementary Fig. S1. The leakiness score is the ratio of the average intensities

between the middle rectangle and the upper rectangle. The leakiness score is then normalized to the baseline value at T0 before treatment.

Ussing chambers

Ussing chamber experiments were conducted at Bioptra (ReproCell Europe Ltd) following the authors' study design. Healthy colon tissue was sourced from surgical residual tissue in accordance with Bioptra tissue protocol TPS-001. All tissues were inspected upon arrival, and any tissue deemed to be macroscopically diseased/necrotic was rejected. Once mounted in the Ussing chamber, any tissues with a potential difference (PD) value greater than -1.0 mV and/or a resistance value of less than $20 \Omega \text{ cm}^2$ were also excluded. The voltage clamp and Ussing chamber system were set up and allowed to reach approximately 37°C over a period of at least 30 minutes. The PD of the electrodes and the resistance due to the physiological saline solution (PSS) was offset prior to the experiment. The mucosa was dissected free from the submucosa and smooth muscle of the colon under a dissection microscope. A total of 12 sections per donor were stretched onto the Ussing sliders (slider area 0.5 cm^2) and clamped onto the sliders. The sliders were then inserted into the Ussing chambers and 5 mL of PSS was added to both sides of the tissue, aerated with 95% $\text{O}_2/5\%$ CO_2 gas mix and maintained at approximately 37°C . The tissues were allowed to equilibrate for approximately 15 minutes at 37°C , in open circuit conditions, and an assessment of the PD of the tissue was then made. Following the equilibration period, the tissue was short circuited by clamping the voltage at zero, allowing the short circuit current (Isc) to be recorded. To determine the resistance of the tissue, and therefore determine whether it was intact, the voltage was changed from 0 to 1 mV for 2 seconds every 60 seconds. The change in Isc produced by this change in voltage was used to determine the resistance using Ohm's law, Resistance = Voltage/delta Current ($R = V/\Delta I$). The tissue was then left to equilibrate in voltage clamp conditions over a period of 15 minutes, representing $t = 0$ for studies described below.

For examination of the effects of pertuzumab and lumretuzumab on chloride transport, changes in Isc were recorded over time after the sequential addition of mAbs, heregulin and channel activators on the basolateral side as follows: Vehicle, pertuzumab, or lumretuzumab ($0.03\text{--}300 \mu\text{g/mL}$) were added at $t = 0$, here-

gulin (100 ng/mL) at $t = 15$ minutes, forskolin ($10 \mu\text{mol/L}$) at $t = 35$ minutes, and carbachol ($100 \mu\text{mol/L}$) at $t = 95$ minute for 60 minutes. Forskolin and carbachol activate chloride secretion in the human gut by increasing cAMP levels and intracellular calcium concentrations, respectively.

Statistical analysis of *in vitro* studies

Statistical analysis was carried out by ANOVA using Dunnett multiple comparison test at 95% confidence interval and an alpha of 0.05. Treatments were analyzed against the vehicle control of similar conditions unless otherwise stated in the figure legends; *P* values were adjusted to account for multiple comparisons.

Results

Clinical safety

In the course of a phase Ib study evaluating the safety and tolerability of a triple combination consisting of lumretuzumab, pertuzumab, and paclitaxel in patients with metastatic breast cancer, all 35 patients experienced at least one episode of diarrhea (Table 1, cohorts 1–3). The first 2 patients treated experienced persistent grade 3 diarrhea considered dose-limiting toxicities (Table 1, cohort 1, see also Mirschberger and colleagues (5)). As risk mitigation to decrease the occurrence and severity of diarrhea in patients, the dose of lumretuzumab was reduced to 500 mg (Table 1, cohort 2). In a subsequent cohort of patients, lumretuzumab was maintained at 500 mg dose, the 820 mg initial dose of pertuzumab (cycle 1, day 1) was reduced to 420 mg, and patients received loperamide prophylactically at cycle 1, day 1 or day 2 of treatment (Table 1, cohort 3). The risk mitigations implemented slightly improved the safety profile by reducing the incidence of grade 3 diarrhea events from 100% in cohort 1 (2/2 patients) to 50% in cohort 2 (10/20 patients) and 30% in cohort 3 (4/13 patients, including one grade 4). A similar trend was noted for the incidence rate of hospitalization due to diarrhea toxicity and for the rate of patients who discontinued study due to diarrhea (Table 1). On the other hand, the rate of grade 2 diarrhea and the time spent by patients on diarrhea during the treatment remained similar between cohorts (Table 1).

While the contribution of each drug in the development of the diarrhea toxicity could not be fully characterized based on the safety information from the 35 patients treated, the following clinical evidence indicated that paclitaxel may not play a

Table 1. Overview of the diarrhea toxicity profile

	Diarrhea toxicity profile overview		
	No. of patients (%)		
	Cohort 1 <i>N</i> = 2	Cohort 2 <i>N</i> = 20	Cohort 3 <i>N</i> = 13
Any diarrhea (all grades)	2 (100)	20 (100)	13 (100)
Grade 1 ^a	0 (0)	2 (10)	2 (15)
Grade 2 ^a	0 (0)	8 (40)	7 (35)
Grade 3 ^a	2 (100)	10 (50)	3 (23)
Grade 4 ^a	0 (0)	0 (0)	1 (8)
Hospitalization due to diarrhea	1 (50)	4 (20)	1 (8)
Study discontinuation due to diarrhea	1 (50)	2 (10)	3 (23)
% time spent on diarrhea during treatment	77%	96%	88%

NOTE: Lumretuzumab and pertuzumab were administered every 3 weeks. Paclitaxel was administered weekly at a dose of 80 mg/m^2 .

Cohort 1: 1,000 mg lumretuzumab dose, 840 mg pertuzumab dose on day 1 of cycle 1, and 420 mg on day 1 of each subsequent cycle.

Cohort 2: 500 mg lumretuzumab dose, 840 mg pertuzumab dose on day 1 of cycle 1, and 420 mg on day 1 of each subsequent cycle.

Cohort 3: 500 mg lumretuzumab dose and 420 mg pertuzumab dose from cycle 1.

^aHighest grade.

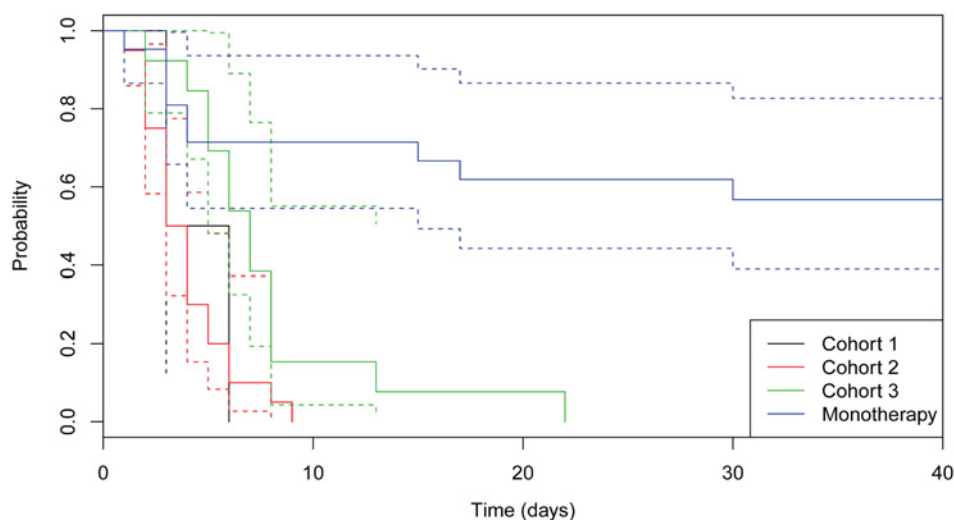


Figure 1.

Probability of being diarrhea free during lumretuzumab monotherapy and combination therapy with pertuzumab. Kaplan-Meier curves and confidence intervals (dotted lines) of the probability of being diarrhea free (any grade), as a function of duration of treatment. Patients are stratified by the treatment received: lumretuzumab monotherapy 2,000 mg dose, blue line (study NCT01482377, $N = 26$ patients, for details of the study see, ref. (6) or in combination with pertuzumab and paclitaxel (study NCT01918254, cohort 1 black line, cohort 2 red line, and cohort 3 green line). Cohort 1: 1,000 mg lumretuzumab dose ($N = 2$ patients); cohort 2: 500 mg lumretuzumab dose ($N = 20$ patients); cohort 3: 500 mg lumretuzumab dose at a reduced dose of pertuzumab at cycle 1 ($N = 13$ patients).

major role, while the combination of the two anti-HER antibodies resulted in significantly enhanced toxicity. In cohort 2, three patients experienced persistent and worsening grade 2/3 diarrhea in intensity while paclitaxel administration was stopped after only 3 administrations, and pertuzumab and lumretuzumab administrations maintained for additional cycles (Supplementary Fig. S2A–S2C). One patient in cohort 3 who experienced diarrhea up to grade 3 at the third cycle, and received only a fraction of the planned pertuzumab dose at the fourth cycle 4 (16%) and the fifth cycle 5 (9%) due to infusion-related reactions (IRR) permanently stopped pertuzumab at the fifth cycle and had no recurrence of diarrhea during seven subsequent cycles with lumretuzumab combined with paclitaxel (Supplementary Fig. S2D). Finally, omission of the pertuzumab loading dose combined with a prophylactic loperamide administration decreased the grade 3 diarrhea incidence rate to 30% in cohort 3 (Table 1).

The early onset of diarrhea in the triple combination therapy was further compared with lumretuzumab monotherapy using the Kaplan-Meier curve estimating the probability of being diarrhea free as a function of days since the initiation of the treatment, i.e., first dose (Fig. 1). The analysis revealed that while the median time of onset of diarrhea of any grade in patients treated with lumretuzumab single agent (2000 mg) was 57 days, the median time with the triple combination with paclitaxel, pertuzumab, and lumretuzumab (1,000 mg in cohort 1 or 500 mg in cohorts 2–3) was, respectively, 4.5, 3.5, and 7 days in the three cohorts.

Although these clinical results suggested an additive and exacerbated effect of lumretuzumab and pertuzumab in the development of the gastrointestinal toxicity compared with single agents, the contribution of each drug and the mechanism underlying the diarrhea toxicity via the HER family signaling pathway remained unclear and prompted us to establish an

in vitro strategy to address the potential effects of antibody-mediated targeting of HER2 and HER3 on intestinal epithelial cell integrity and function.

Cellular model for mechanistic investigations of gastrointestinal toxicity

The human colorectal adenocarcinoma-derived colonocyte cell line Caco2 was selected amongst other commonly used intestinal cell lines based on the relative expression of HER1, HER2, and HER3 that resembles the normal human intestine profile (Fig. 2A and B). Caco2 cells form monolayers of epithelium and constitute a validated cell model for drug transport, permeability, and toxicity studies (23).

Target engagement was verified by exposing Caco2 cells to pertuzumab and/or lumretuzumab in the presence of the HER3 ligand heregulin followed by immunoblot analysis of target receptors and downstream signaling molecules. Binding of pertuzumab to HER2 blocks dimerization with other HER receptors and allows ligand-independent phosphorylation of HER2, leading to its degradation by the proteasome (24, 25). Accordingly, exposure of Caco2 cells to pertuzumab resulted in increased phosphorylation and degradation of HER2 and robust downregulation of HER2-dependent phosphorylation of HER3 (Fig. 2C). Binding of lumretuzumab to HER3 blocks HER2/HER3 dimer formation and prevents ligand-dependent phosphorylation of both receptors (5, 26), as also observed in Caco2 cells exposed to lumretuzumab (Fig. 2C). Combination of pertuzumab and lumretuzumab showed enhanced effects on HER2 and HER3 phosphorylation, and neither treatment significantly altered HER1 phosphorylation and stability under these conditions (Fig. 2C). Blockage of HER2/HER3 downstream signaling by pertuzumab and lumretuzumab in Caco2 cells was confirmed by a strong decrease in phosphorylated AKT and ERK1/2, an effect enhanced by the combination

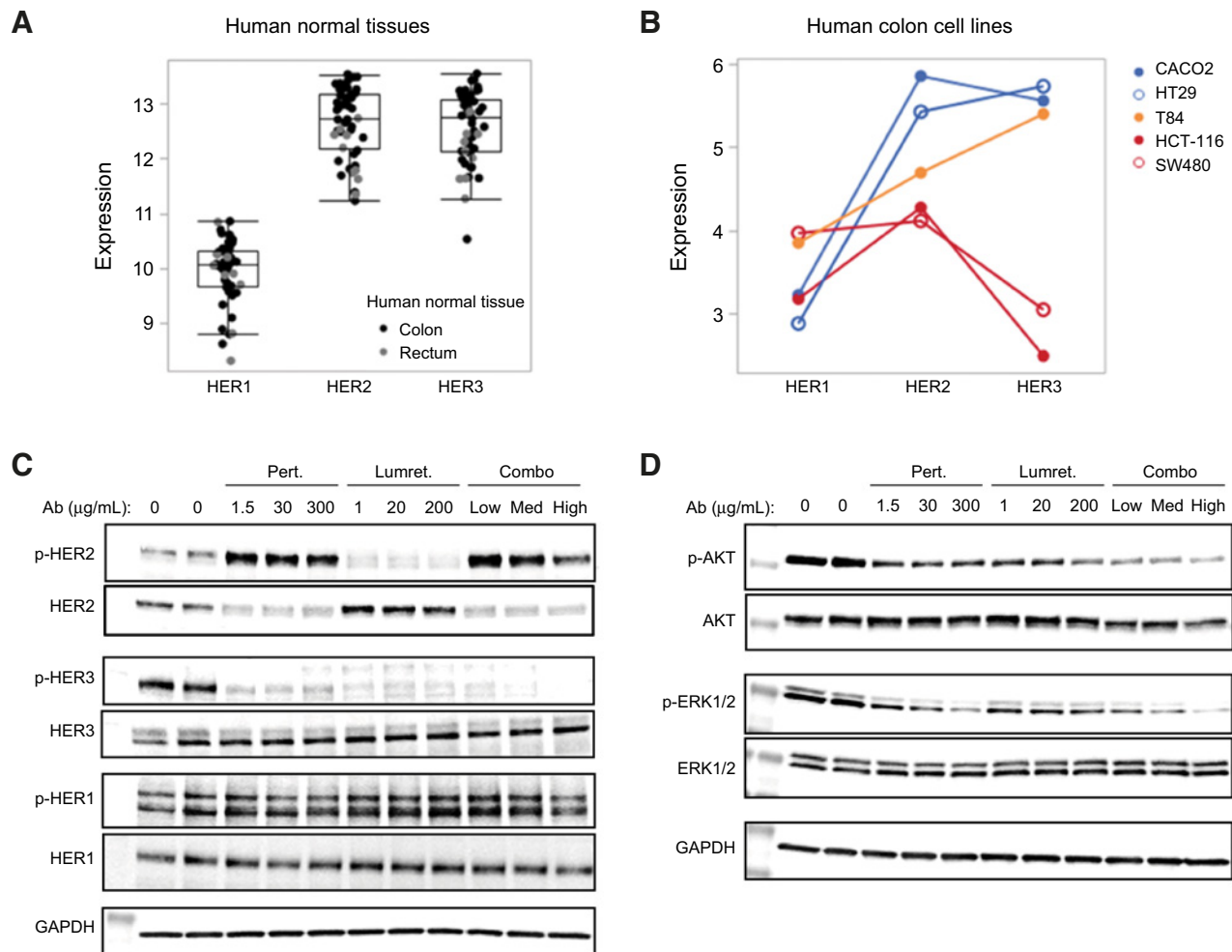


Figure 2. Target engagement by pertuzumab and lumretuzumab in Caco2 colonocyte cells. **A** and **B**, Gene expression (log RPKM values) of HER family members HER1, HER2, and HER3 in **(A)** human normal colorectal samples from The Cancer Genome Atlas (TCGA) and **(B)** in five selected colon cell lines from the Roche internal CELLO dataset: Caco2 and HT-29 best mimic the gene expression profile of the human normal samples, while the other samples showing comparatively lower expression in HER2 and/or HER3. **C**, Immunoblot analysis showing the regulation of HER2 and HER3 protein level and phosphorylation by pertuzumab and lumretuzumab, individually and combined. **D**, Immunoblot analysis showing that phosphorylation of AKT and ERK1/2 is reduced by exposure to pertuzumab and lumretuzumab in a dose-dependent and additive manner. Ab, therapeutic antibody; pert, pertuzumab; lumret, lumretuzumab. Low, med, high: ratio pertuzumab:lumretuzumab in combination treatment was 1.5:1, 30:20, and 300:200 µg/mL respectively. Representative of $n = 2$.

treatment (Fig. 2D). These data demonstrate full target engagement and inhibition of HER2 and HER3 and validate the use of Caco2 cells for mechanistic studies.

Pertuzumab and lumretuzumab preserve intestinal epithelial barrier integrity

We investigated the effect of HER2/HER3-targeting therapy on intestinal cell proliferation and viability by exposing subconfluent Caco2 cells to pertuzumab and lumretuzumab over a period of 5 days followed by measurement of intracellular ATP and dead cell protease release (Fig. 3A). Whereas the proapoptotic toxic reference compound staurosporine inhibited proliferation and viability and caused plasma membrane disruption as indicated by protease release in the supernatant, pertuzumab and lumretuzumab, used as single or combination treatments,

appeared innocuous in both assays even at suprapharmacological concentrations (Fig. 3B and C).

To verify whether the epithelial barrier function was preserved in the context of HER2/HER3 inhibition, Caco2 cells were grown in microfluidic chambers until formation of a tight epithelium along a collagen I matrix (21) and exposed to increasing concentrations of pertuzumab, lumretuzumab, and their combination for up to 48 hours (Fig. 3D–F). Drug-induced epithelial barrier disruption was measured by quantifying the passage of a fluorescently labeled dextran from the luminal side into the collagen matrix (Fig. 3D). In contrast to the chemotherapeutic agent paclitaxel, pertuzumab, and lumretuzumab maintained the barrier property of intestinal cells intact at all concentrations tested in combination (Fig. 3E and F). Overall, the data suggest that anti-HER2/3 combination

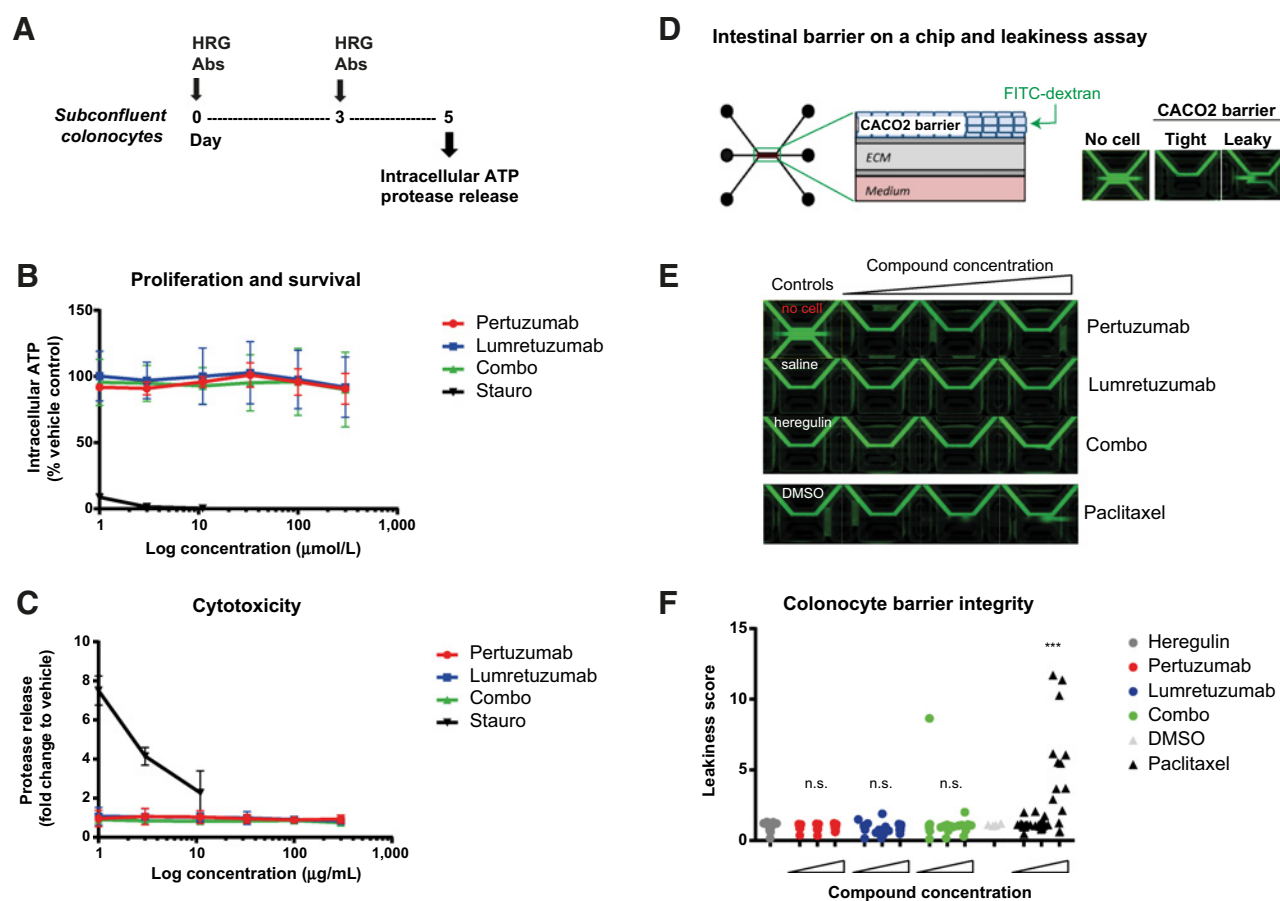


Figure 3.

Pertuzumab and lumretuzumab preserve intestinal epithelial barrier integrity *in vitro*. **A**, Schematic representation of cell proliferation and survival assay timeline in Caco2 cells. HRG, heregulin; Abs, antibodies. **B**, Measurement of intracellular ATP showing a negative effect of staurosporine (stauro) and no detectable effect of pertuzumab and lumretuzumab on cell proliferation and survival. **C**, Measurement of dead cell protease release showing the dose-dependent plasma membrane leakage induced by staurosporine and the innocuous effect of pertuzumab and lumretuzumab. X axis represents the pertuzumab concentrations. The pertuzumab:lumretuzumab ratio was kept at 3:2 for single and combination (combo) treatment. Staurosporine concentrations were 0.1, 1, and 10 $\mu\text{mol/L}$. The reduction in dead protease at 1 and 10 $\mu\text{mol/L}$ compared with 0.1 $\mu\text{mol/L}$ is presumably a consequence of rapid cell death and protease degradation at time of measurement. **D**, Schematic representation of epithelial barrier formation and leakage assay using Caco2 cells grown in a microfluidic OrganoPlate. Diffusion of a fluorescently labeled dextran (FITC-dextran) from the Caco2 lumen into the extracellular matrix (ECM) and cell-free medium channel indicates Caco2 barrier disruption. **E** and **F**, Measurement of epithelial barrier integrity after 48 hours of treatment with compounds showing the preservation of barrier integrity by pertuzumab and lumretuzumab and induction of leakiness by paclitaxel at the highest tested concentration. Selected representative images are displayed in **E** and quantification of FITC-dextran leakiness across the epithelial barrier (leakiness score, see material and methods) is shown in **F**. Concentrations and number of replicates were for pertuzumab 1.5, 30, and 300 $\mu\text{g/mL}$ ($n = 6$ per concentration), for lumretuzumab 1, 20, and 200 $\mu\text{g/mL}$ ($n = 7$ per concentration), for pertuzumab:lumretuzumab combination (combo) 1.5:1, 30:20, and 300:200 $\mu\text{g/mL}$ ($n = 7$ per combination), for paclitaxel 2, 20, and 200 nmol/L ($n = 14$ per concentration), for heregulin (5 ng/mL , $n = 13$), and DMSO ($n = 4$). n.s., not statistically significant compared with heregulin control; ***, $P < 0.0001$ compared with DMSO control; ANOVA with Dunnett multiple comparisons test.

treatment with pertuzumab and lumretuzumab does not alter intestinal epithelial cell proliferation nor epithelial barrier function.

Pertuzumab and lumretuzumab modulate chloride secretion in human colon mucosa

In the normal colon, sodium absorption and chloride secretion are stimulated by secondary messengers such as calcium and cyclic AMP (cAMP; ref. 27). Previous *in vitro* studies in the T84 colonocyte cell line described the negative modulation of calcium-induced chloride transport by EGF/HER1 and heregulin/HER2-HER3 signaling (28–31). However, modulation of

calcium and cAMP-induced chloride secretion by pharmacologic inhibition of HER2/3 has not been reported. Here, we directly assessed the effect of pertuzumab and lumretuzumab on chloride secretion by using freshly isolated human colon mucosa explants mounted in Ussing chambers, a method that measures the short circuit current as an indicator of net ion transport taking place across an epithelium. Heregulin, lumretuzumab, and pertuzumab were added on the basolateral side where HER2 and HER3 are localized, followed by cAMP and calcium induction via forskolin (FSK) and carbachol (Cch), respectively (Fig. 4A). First, this assay revealed the downregulation of both cAMP and calcium-activated chloride secretion by

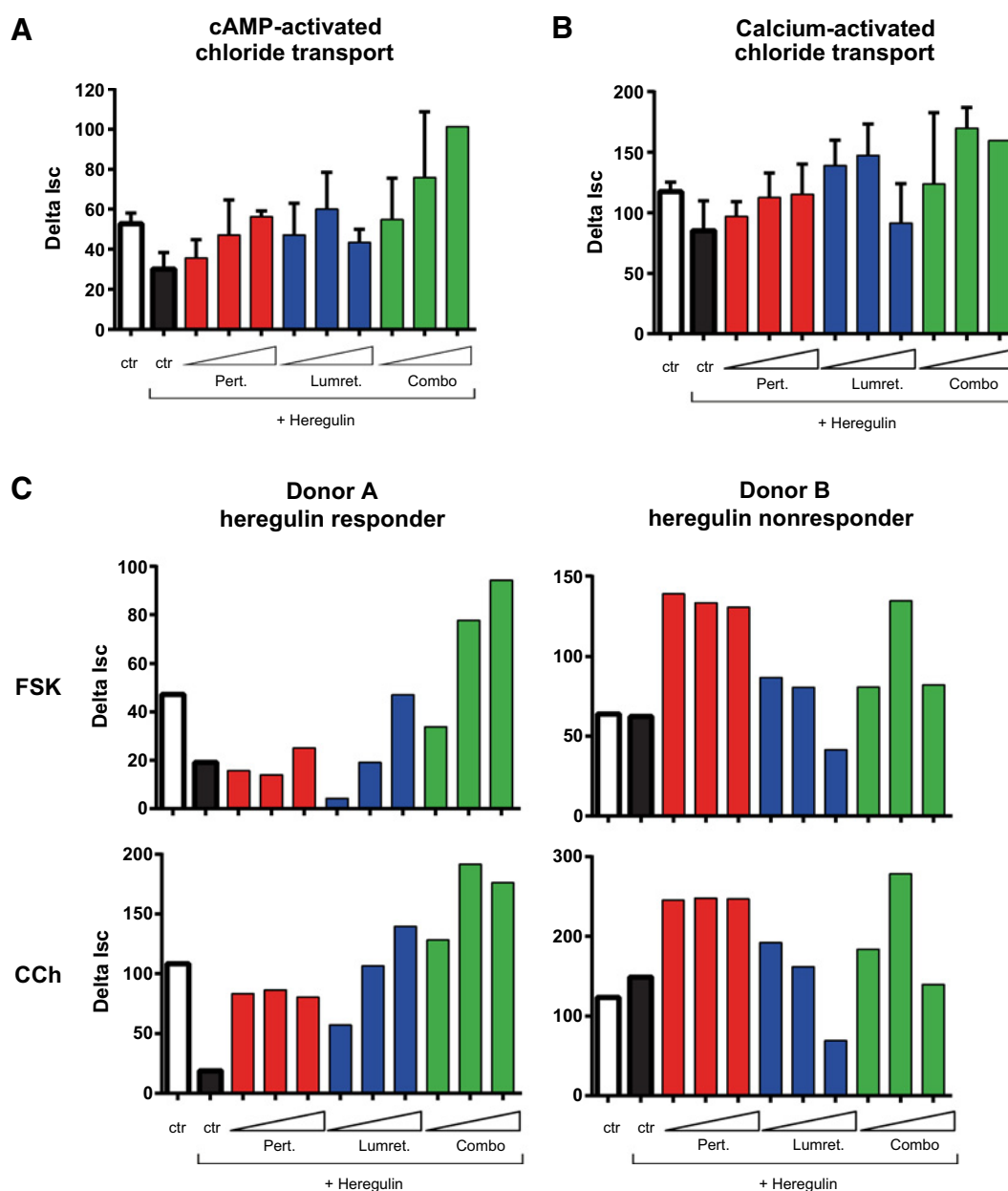


Figure 4.

Pertuzumab and lumretuzumab positively modulate chloride transport in fresh human colon mucosa. **A** and **B**, Graphs showing the effects of pertuzumab and lumretuzumab, alone and in combination, on the plateau response of cAMP-induced chloride transport via forskolin (**A**) or peak response of calcium-induced chloride transport via carbachol (**B**) in tissues that have been exposed to heregulin for 20 minutes where indicated. Concentrations and number of replicates were as follows: Heregulin 100 ng/mL control ($N = 4$), pertuzumab and lumretuzumab as single agent 0.3 $\mu\text{g/mL}$ ($N = 4$), 3 $\mu\text{g/mL}$ ($N = 4$) and 10 $\mu\text{g/mL}$ ($N = 2$), combination pertuzumab: lumretuzumab (combo) 0.3:0.3 $\mu\text{g/mL}$ ($N = 3$), 3:3 $\mu\text{g/mL}$ ($N = 3$) and 10:10 $\mu\text{g/mL}$ ($N = 1$), vehicle control (saline, $N = 4$). Data represent means and SEM. **C**, Interdonor variability as exemplified by donor A, a heregulin responder showing dose response of pertuzumab and lumretuzumab and additive effect of combination treatment, and donor B, a heregulin nonresponder showing plateau response of pertuzumab and irregular effect of combination treatment. I_{sc}, short circuit current; FSK, forskolin; CCh, carbachol.

heregulin in fresh human colon mucosa (Fig. 4B and C). Second, attenuation of cAMP and calcium-induced chloride secretion by heregulin was reversed in the presence of pertuzumab or lumretuzumab in a concentration-dependent manner. Third, the combination of lumretuzumab and pertuzumab at concentrations up to 10 $\mu\text{g/mL}$ had an enhanced effect

compared with single agents (Fig. 4B and C). Chloride secretion above vehicle control was sometimes noticeable, particularly in the presence of pertuzumab, suggesting heregulin-independent effects, likely due to inhibition of HER1:HER2 dimerization. It is worth mentioning that high donor-to-donor variability was observed in the amplitude of heregulin

response, with more robust heregulin effects generally associated with clearer dose-dependent upregulation of chloride secretion by lumretuzumab and combination treatment, as exemplified with two selected donors (Fig. 4D). Donor-to-donor differences may be reflective of clinical situations where interindividual differences in enterocyte function and HER activation are expected.

Discussion

Here, we report on preclinical studies to investigate potential mechanisms explaining the high incidence and severity of diarrhea observed in the course of a phase Ib clinical study, NCT01918254, evaluating the safety of the targeted combination therapy consisting of the anti-HER3 lumretuzumab and anti-HER2 pertuzumab, administered along with the chemotherapeutic agent paclitaxel in patients with metastatic breast cancer (4, 32). Overall, investigations suggest that the mechanism of exacerbated diarrhea observed in patients treated with the triple combination therapy involves an upregulation of HER2/HER3-controlled chloride channel activity mediated by lumretuzumab and pertuzumab, without manifestation of tissue damages caused by the two antibodies.

Interestingly, the standard-of-care diarrhea treatment with loperamide, that is systematically prescribed for patients with chemotherapy-induced diarrhea such as paclitaxel to decrease the intestinal motility by directly affecting the smooth muscle of the intestine (1, 33), did not result in the expected benefit in this study. In particular in cohorts 2 and 3, despite the administration of loperamide, at first signs of diarrhea or administered prophylactically, the early onset (less than 1 week, median time) and long duration of diarrhea (up to 96% of time under treatment) continued to impact the quality of life of patients during the entire duration of the study treatment.

The proposed risk mitigation of toxicities associated with paclitaxel included as well a dose delay, a dose reduction, or permanent discontinuation of the chemotherapy. Unfortunately, even the permanent discontinuation of paclitaxel did not appear to improve diarrhea as shown in 3 patients who experienced ongoing grade 2/3 diarrhea toxicity despite discontinuation of paclitaxel. On the other hand, the permanent discontinuation of pertuzumab treatment in 1 patient who experienced diarrhea grade 3 resulted in the full resolution of the toxicity with no reoccurrence while the patient was still treated with paclitaxel/lumretuzumab combination. A similar observation was reported from a phase Ib study, where the combination of lumretuzumab plus the chemotherapies carboplatin and paclitaxel was considered well tolerated, and though 75% of patients experienced diarrhea, none were grade 3/4 diarrhea (34). In the present study, the reduction of lumretuzumab dose from 1,000 mg to 500 mg combined with a reduction of the first pertuzumab dose in cohort 3 likely contributed to the observed reduction of the incidence of grade 3 toxicity and delayed the onset, suggesting a key contribution of the inhibitory pathway of HER family members in the etiology of the observed diarrhea.

The differences in the clinical manifestations of diarrhea associated with paclitaxel and lumretuzumab/pertuzumab suggest that cytotoxic chemotherapeutic drugs and HER-targeting antibodies act on intestinal barrier function via divergent

mechanisms. At the cellular level, paclitaxel binds to microtubules and, similar to other chemotherapeutic drugs, is believed to directly damage intestinal cells, leading to disruption of enterocyte brush border, intestinal barrier integrity, and fluid balance (35). Using an *in vitro* intestinal barrier model and integrity assay, we confirmed that exposure of colonocytes to paclitaxel can lead to barrier leakiness. On the contrary, pertuzumab and lumretuzumab did not disrupt the intestinal barrier integrity even when applied in combination and at supra-pharmacologic concentrations shown to fully inhibit the HER2:3 pathway in the same *in vitro* model. Instead, our experiments performed with fresh human colon mucosa revealed that heregulin is a negative regulator of cAMP- and calcium-induced chloride secretion, and that pertuzumab and lumretuzumab can efficiently revert heregulin-dependent chloride channel activity, thereby increasing chloride secretion. Moreover, an additive effect of pertuzumab and lumretuzumab combination treatment was observed, in accordance with the diarrhea profile reported in the clinical study. Altogether, the data presented here substantiate the hypothetical induction of secretory diarrhea by lumretuzumab and pertuzumab combination therapy as a consequence of direct modulation of chloride channels and demonstrate that full inhibition of the HER family signaling pathway may account for the severe diarrhea observed in the clinical setting.

This is the first study combining safety clinical data on diarrhea related to HER-targeting therapy and experimental verification of the mechanistic hypothesis. Importantly, the experimental evidence evokes the relevance to consider anti-diarrheal drugs that target cAMP and calcium-activated chloride channels as alternative to or in parallel with conventional antidiarrheal medication such as loperamide. Given its mechanism of action, the chloride channel inhibitor crofelemer, currently FDA approved to relieve symptoms of diarrhea in HIV-positive patients taking antiretroviral therapy, may provide a more targeted approach at preventing diarrhea in patients receiving HER-targeted therapies and is currently being investigated in a phase II study with metastatic breast cancer patients treated with trastuzumab, pertuzumab, and chemotherapy (36). Such targeted strategy for diarrhea risk management may allow the maintenance of the most efficacious doses and combinations of anti-HER antibodies and hopefully contribute to reach a therapeutic window with increased benefits for more cancer patients.

Disclosure of Potential Conflicts of Interest

A. Moisan, W. Jacob and M. Weisser have ownership interest (including patents) in Roche stocks. No potential conflicts of interest were disclosed by the other authors.

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References

- Pessi MA, Zilembo N, Haspinger ER, Molino L, Di Cosimo S, Garassino M, et al. Targeted therapy-induced diarrhea: a review of the literature. *Crit Rev Oncol Hematol* 2014;90:165–79.
- Ratner M. FDA deems in vitro data on mutations sufficient to expand cystic fibrosis drug label. *Nat Biotechnol* 2017;35:606.
- Bhatia SN, Ingber DE. Microfluidic organs-on-chips. *Nat Biotechnol* 2014;32:760–72.
- Schneeweiss A, Park-Simon TW, Albanell J, Lassen U, Cortes J, Dieras V, et al. Phase Ib study evaluating safety and clinical activity of the anti-HER3 antibody lumretuzumab combined with the anti-HER2 antibody pertuzumab and paclitaxel in HER3-positive, HER2-low metastatic breast cancer. *Invest New Drugs* 2018. <https://doi.org/10.1007/s10637-018-0562-4>
- Mirschberger C, Schiller CB, Schraml M, Dimoudis N, Friess T, Gerdes CA, et al. RG7116, a therapeutic antibody that binds the inactive HER3 receptor and is optimized for immune effector activation. *Cancer Res* 2013;73:5183–94.
- Meulendijks D, Jacob W, Martinez-Garcia M, Taus A, Lolkema MP, Voest EE, et al. First-in-human phase I study of lumretuzumab, a glycoengineered humanized anti-HER3 monoclonal antibody, in patients with metastatic or advanced HER3-positive solid tumors. *Clin Cancer Res* 2016;22:877–85.
- Meulendijks D, Jacob W, Voest EE, Mau-Sorensen M, Martinez-Garcia M, Taus A, et al. Phase Ib study of lumretuzumab plus cetuximab or erlotinib in solid tumor patients and evaluation of HER3 and heregulin as potential biomarkers of clinical activity. *Clin Cancer Res* 2017;23:5406–15.
- Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127–37.
- Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nat Rev Cancer* 2009;9:463–75.
- Jones SE, Erban J, Overmoyer B, Budd GT, Hutchins L, Lower E, et al. Randomized phase III study of docetaxel compared with paclitaxel in metastatic breast cancer. *J Clin Oncol* 2005;23:5542–51.
- Gianni L, Llado A, Bianchi G, Cortes J, Kellokumpu-Lehtinen PL, Cameron DA, et al. Open-label, phase II, multicenter, randomized study of the efficacy and safety of two dose levels of Pertuzumab, a human epidermal growth factor receptor 2 dimerization inhibitor, in patients with human epidermal growth factor receptor 2-negative metastatic breast cancer. *J Clin Oncol* 2010;28:1131–7.
- Swain SM, Kim SB, Cortes J, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel for HER2-positive metastatic breast cancer (CLEOPATRA study): overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. *Lancet Oncol* 2013;14:461–71.
- Swain SM, Schneeweiss A, Gianni L, Gao JJ, Stein A, Waldron-Lynch M, et al. Incidence and management of diarrhea in patients with HER2-positive breast cancer treated with pertuzumab. *Ann Oncol* 2017;28:761–8.
- Frey MR, Brent Polk D. ErbB receptors and their growth factor ligands in pediatric intestinal inflammation. *Pediatr Res* 2014;75:127–32.
- Loriot Y, Perlemuter G, Malka D, Penault-Llorca F, Boige V, Deutsch E, et al. Drug insight: gastrointestinal and hepatic adverse effects of molecular-targeted agents in cancer therapy. *Nat Clin Pract Oncol* 2008;5:268–78.
- Hirsh V, Blais N, Burkes R, Verma S, Croitoru K. Management of diarrhea induced by epidermal growth factor receptor tyrosine kinase inhibitors. *Curr Oncol* 2014;21:329–36.
- Van Sebillie YZ, Gibson RJ, Wardill HR, Bowen JM. ErbB small molecule tyrosine kinase inhibitor (TKI) induced diarrhea: chloride secretion as a mechanistic hypothesis. *Cancer Treat Rev* 2015;41:646–52.
- Lassen UN, Ruiperez AC, Fleitas T, Meulendijks D, Schellens J, Lolkema M, et al. 444O – Phase IB trial of RG7116, a glycoengineered monoclonal antibody targeting HER3, in combination with cetuximab or erlotinib in patients with advanced/metastatic tumors of epithelial cell origin expressing HER3 protein. *Ann Oncol* 2014;25:iv147–iv.
- Jankowitz RC, Abraham J, Tan AR, Limentani SA, Tierno MB, Adamson LM, et al. Safety and efficacy of neratinib in combination with weekly paclitaxel and trastuzumab in women with metastatic HER2-positive breast cancer: an NSABP Foundation Research Program phase I study. *Cancer Chemother Pharmacol* 2013;72:1205–12.
- Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 2008;5:621–8.
- Trietsch SJ, Naumovska E, Kurek D, Setyawati MC, Vormann MK, Wilschut KJ, et al. Membrane-free culture and real-time barrier integrity assessment of perfused intestinal epithelium tubes. *Nat Commun* 2017;8:262.
- Duska LR, Penson R, Supko JG, Finkelstein DM, Makastorsis T, Gallagher J, et al. A phase I study of continuous infusion doxorubicin and paclitaxel chemotherapy with granulocyte colony-stimulating factor for relapsed epithelial ovarian cancer. *Clin Cancer Res* 1999;5:1299–305.
- Hubatsch I, Ragnarsson EG, Artursson P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat Protoc* 2007;2:2111–9.
- Goltsov A, Deeni Y, Khalil HS, Soiminen T, Kyriakidis S, Hu H, et al. Systems analysis of drug-induced receptor tyrosine kinase reprogramming following targeted mono- and combination anti-cancer therapy. *Cells* 2014;3:563–91.
- Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* 2004;5:317–28.
- Peess C, von Proff L, Goller S, Andersson K, Gerg M, Malmqvist M, et al. Deciphering the stepwise binding mode of HRC1beta to HER3 by surface plasmon resonance and interaction map. *PLoS One* 2015;10:e0116870.
- Viswanathan VK, Hodges K, Hecht G. Enteric infection meets intestinal function: how bacterial pathogens cause diarrhea. *Nat Rev Microbiol* 2009;7:110–9.
- Mroz MS, Keely SJ. Epidermal growth factor chronically upregulates Ca²⁺-dependent Cl⁻ conductance and TMEM16A expression in intestinal epithelial cells. *J Physiol* 2012;590:1907–20.

29. O'Mahony F, Toumi F, Mroz MS, Ferguson G, Keely SJ. Induction of Na⁺/K⁺/2Cl⁻ cotransporter expression mediates chronic potentiation of intestinal epithelial Cl⁻ secretion by EGF. *Am J Physiol Cell Physiol* 2008;294:C1362–70.
30. Keely SJ, Barrett KE. ErbB2 and ErbB3 receptors mediate inhibition of calcium-dependent chloride secretion in colonic epithelial cells. *J Biol Chem* 1999;274:33449–54.
31. Uribe JM, Gelbmann CM, Traynor-Kaplan AE, Barrett KE. Epidermal growth factor inhibits Ca(2+)-dependent Cl⁻ transport in T84 human colonic epithelial cells. *Am J Physiol* 1996;271:C914–22.
32. Martinez-Garcia; AST-WP-SJAULCVDMMCSFMJMCM. Phase Ib study evaluating safety and clinical activity of lumretuzumab combined with pertuzumab and paclitaxel in HER3-positive, HER2-low metastatic breast cancer. *Breast Cancer Res* 2017;under review.
33. Andreyev J, Ross P, Donnellan C, Lennan E, Leonard P, Waters C, et al. Guidance on the management of diarrhea during cancer chemotherapy. *Lancet Oncol* 2014;15:e447–60.
34. Cejalvo JM, Fleitas T, Felip E, Mendivil AN, Martinez-Garcia M, Taus A, et al. A phase Ib study of lumretuzumab, a glycoengineered monoclonal antibody targeting HER3, in combination with carboplatin and paclitaxel as 1st-line treatment in patients with squamous non-small cell lung cancer. *Ann Oncol* 2016;27:372P–P.
35. Gibson RJ, Keefe DM. Cancer chemotherapy-induced diarrhea and constipation: mechanisms of damage and prevention strategies. *Support Care Cancer* 2006;14:890–900.
36. Gao JJ, Tan M, Pohlmann PR, Swain SM. HALT-D: a phase II evaluation of crofelemer for the prevention and prophylaxis of diarrhea in patients with breast cancer on pertuzumab-based regimens. *Clin Breast Cancer* 2016; 17:76–8.