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Fluorescently labelled vedolizumab to visualise drug distribution and mucosal target cells in inflammatory bowel disease

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ABSTRACT

Objective Improving patient selection and development of biological therapies such as vedolizumab in IBD requires a thorough understanding of the mechanism of action and target binding, thereby providing individualised treatment strategies. We aimed to visualise the macroscopic and microscopic distribution of intravenous injected fluorescently labelled vedolizumab, vedo-800CW, and identify its target cells using fluorescence molecular imaging (FMI).

Design Forty three FMI procedures were performed, which consisted of macroscopic in vivo assessment during endoscopy, followed by macroscopic and microscopic ex vivo imaging. In phase A, patients received an intravenous dose of 4.5 mg, 15 mg vedo-800CW or no tracer prior to endoscopy. In phase B, patients received 15 mg vedo-800CW preceded by an unlabelled (sub)therapeutic dose of vedolizumab.

Results FMI quantification showed a dose-dependent increase in vedo-800CW fluorescence intensity in inflamed tissues, with 15 mg (153.7 au (132.3–163.7)) as the most suitable tracer dose compared with 4.5 mg (55.3 au (33.6–78.2)) (p=0.0002). Moreover, the fluorescence signal decreased by 61% when vedo-800CW was administered after a therapeutic dose of unlabelled vedolizumab, suggesting target saturation in the inflamed tissue. Fluorescence microscopy and immunostaining showed that vedolizumab penetrated the inflamed mucosa and was associated with several immune cell types, most prominently with plasma cells.

Conclusion These results indicate the potential of FMI to determine the local distribution of drugs in the inflamed target tissue and identify drug target cells, providing new insights into targeted agents for their use in IBD.

Trial registration number NCT04112212.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Vedolizumab, prescribed for IBD, inhibits α4β7 integrin and was developed to prevent the migration of α4β7-expressing gut-homing T cells from vessels into the mucosa, thereby preventing inflammation, but recent studies have speculated that the anti-inflammatory effect of vedolizumab is mediated by a wide range of α4β7-expressing immune cells, not just T cells.

⇒ Combining fluorescence molecular imaging (FMI) with fluorescently labelled drugs holds high potential for providing detailed insights into drug distribution and better understanding of the mechanism of action by allowing visualisation of its target cells.

WHAT THIS STUDY ADDS

⇒ Intravenous administration of fluorescently labelled vedolizumab combined with FMI in 43 procedures in patients with IBD enabled visualisation of both in vivo and ex vivo drug distribution, and a clear decrease in fluorescence signal was detected after addition of an unlabelled vedolizumab predose suggesting target saturation.

⇒ Vedolizumab migrated into the inflamed mucosa, bound to the surface of plasma cells and was taken up into the cytoplasm of macrophages and eosinophils.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The ability to localise a drug's distribution and identify its target cells is an essential step towards improved understanding of targeted therapies to personalise treatment options for both IBD and other inflammatory diseases, thereby eventually improving outcome and increasing quality of life.

INTRODUCTION

Biological therapies such as the monoclonal antibody vedolizumab are important options for the treatment of IBD,[1] a group of chronic idiopathic inflammatory disorders that affect the GI tract and consist primarily of Crohn's disease (CD) and UC. Unfortunately, only half of all patients with IBD respond to vedolizumab therapy, and only 39–45% of patients maintain clinical remission.[2] Selecting patients who are suitable for vedolizumab therapy is currently not possible, as no reliable tools are available for predicting response. Moreover,

administering an unsuitable treatment can lead to unnecessary disease burden, adverse effects and high costs,[3] thus highlighting the need to develop clinical tools for optimised treatment and to predict response in individual patients. Several parameters have been investigated as potential indicators of patient outcome, including serological biomarkers, mucosal biomarkers, vedolizumab trough levels, clinical scores and the gut microbiome; however, none has shown a sufficient correlation with the response to vedolizumab therapy in order to be incorporated into the clinical decision-making process.[4–7] In addition, surprisingly little is known regarding drug distribution in the inflamed gut.

Currently, vedolizumab's precise mechanism of action is under debate. Initially, the prevailing theory was that vedolizumab exclusively reduces inflammation by specifically binding to the α4β7 integrin expressed on gut-homing T cells. The resulting integrin-antibody complex blocks T cells from trafficking to the GI mucosa, thereby preventing inflammation.[8–10] Recent studies suggest that a wide range of α4β7-expressing immune cells may be involved in mediating vedolizumab's anti-inflammatory effects.[11–19] However, none of these experimental studies directly visualised the tissue distribution of vedolizumab or its interaction with target cells in the inflamed gut mucosa.

Previously, we used fluorescence molecular imaging (FMI) to visualise the distribution of fluorescent tracers in GI malignancies.[20 21] FMI is a relatively new imaging technique that includes both in vivo fluorescence imaging during endoscopy and ex vivo fluorescence analysis of tissue samples; however, to date this technique has not been used to study drug distribution in inflammatory disease. Here, we performed a phase I clinical trial to assess the feasibility of using FMI after intravenous administration of fluorescently labelled vedolizumab in order to visualise the drug's distribution and identify potential target cells in patients with IBD.

MATERIALS AND METHODS

Study design

This single-centre clinical feasibility trial was performed at the University Medical Centre Groningen (UMCG). The study was conducted in accordance with the Dutch Act on Medical Research Involving Human Subjects (WMO) and the principles of the Declaration of Helsinki (adapted at the 64th WMA General Assembly in Fortaleza, Brazil, 2013). All authors had access to the study data and reviewed and approved the final manuscript. The trial was registered at ClinicalTrials.gov (NCT04112212).

Both vedolizumab-naïve patients with IBD and patients with IBD who received vedolizumab therapy for at least 14 weeks were eligible to participate in the study. Written informed consent was obtained from all patients. To meet the inclusion criteria, patients had to be ≥18 years of age, had an established diagnosis of IBD, a clinical indication for a colonoscopy based on faecal calprotectin levels and/or clinical scoring indices before their first endoscopy procedure, and were considered eligible to receive vedolizumab treatment. We excluded female patients who were pregnant or breast feeding. All study-related procedures are depicted in figure 1.

Good Manufacturing Practice production of vedolizumab-800CW

The fluorescent tracer vedolizumab-800CW (vedo-800CW) was produced by conjugating vedolizumab (Entyvio; Takeda Pharma, Tokyo, Japan) to the IRDye 800CW *N*-Hydroxysuccinimide (NHS) ester near-infrared dye (LI-COR Biosciences, Lincoln, Nebraska) in the UMCG Department of Clinical Pharmacy and Pharmacology's Good Manufacturing Practice (GMP) production unit in accordance with European Union GMP guidelines. A detailed description of the labelling and development process was reported previously.[22]

Patient cohorts

This clinical trial consisted of five distinct patient cohorts and was divided into two phases (A and B), as shown schematically in figure 2. In phase A, 15 vedolizumab-naïve patients were enrolled in a vedo-800CW dose-finding study. Each patient was then assigned to one of the following vedo-800CW dose cohorts (n=5 patients each): 0 mg (serving as a negative control group), 4.5 mg or 15 mg vedo-800CW. Dosage schemes were based on previous results in FMI studies.[23] An interim analysis was conducted in order to evaluate the best dose by assessing various safety parameters and imaging results. In the 4.5 mg group, neither the macroscopic nor microscopic fluorescence signal of vedo-800CW was adequate to visualise the tracer's distribution and detect the target cells using a Zeiss AxioScan Z1 microscope; however, the in vivo images and ex vivo experiments in the 15 mg group provided sufficient results for analysis. Based on this interim analysis, the group that received 15 mg was increased by including 10 additional patients, for a total of 15 patients in this group. In phase B, a dose of 15 mg was then administered to two additional patient cohorts in order to evaluate mucosal saturation of the drug. Predosing patients with unlabelled fluorescence tracer was found to lead to higher target-to-background ratios by preventing off-target uptake in a previous clinical trial by our research group.[23] First, five vedolizumab-naïve patients received a single subtherapeutic dose (75 mg) of unlabelled vedolizumab, followed by a 15 mg dose of vedo-800CW. Next, 13 patients received a 15 mg dose of vedo-800CW after a routine vedolizumab treatment infusion; 12 of these 13 patients received 300 mg vedolizumab in accordance with current guidelines, while the remaining patient received a higher dose of 600 mg due to their body mass index of 61.8. Three patients in the therapeutic dosing group received the tracer after receiving their first dose in the therapeutic vedolizumab regimen; the remaining 10 patients had received at least 14 weeks of vedolizumab treatment prior to receiving vedo-800CW. Unlabelled vedolizumab was administered 1 hour prior to vedo-800CW and both were administered intravenously 2–4 days prior to endoscopy.

Baseline patient characteristics included the Simple Clinical Colitis Activity Index and the Harvey-Bradshaw Index for patients with UC and CD, respectively. In addition, blood and stool samples were collected from all patients and used to perform a complete blood count and to measure plasma C reactive protein and faecal calprotectin levels. Furthermore, prior biological and immunomodulator therapy is depicted in online supplemental table S1.

Macroscopic in vivo imaging

All study-related endoscopy procedures were performed by a gastroenterologist (author WBN) who was experienced in fluorescence endoscopy procedures. A tandem procedure was performed combining high-definition white light endoscopy (HD-WLE) to assess inflammation status with in vivo FMI to visualise the fluorescent signals in select ileocolonic segments in endoscopically assessed inflamed and non-inflamed regions. In vivo FMI was performed by inserting a fibre bundle coupled to the custom-built SurgVision Explorer Endoscopy (SurgVision,

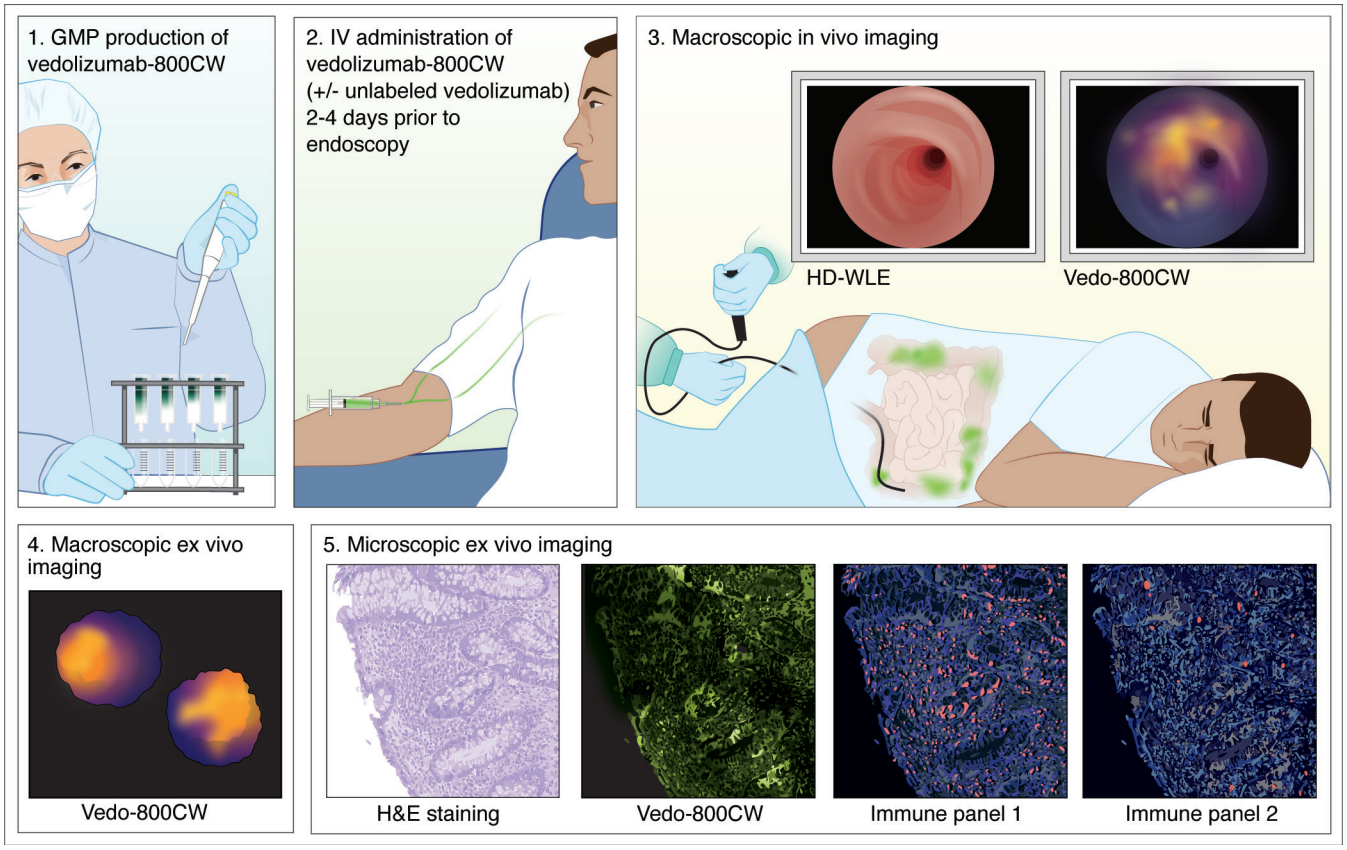

Figure 1 Overview of the procedures included in this study. (1) The fluorescent tracer vedo-800CW was produced in accordance with Good Manufacturing Practice (GMP). (2) Vedo-800CW with or without unlabelled vedolizumab (where applicable) was administered intravenously 2–4 days prior to endoscopy. (3) A tandem in vivo procedure including high-definition white light endoscopy (HD-WLE) and fluorescence molecular endoscopy was performed in order to image the drug's distribution. (4) Fluorescence intensity was quantified ex vivo on formalin-fixed paraffin-embedded (FFPE) blocks in all biopsies. (5) Finally, 4 µm tissue sections were used to assess histopathological inflammation status based on H&E staining, to visualise the microscopic drug distribution and to identify immune target cell types.

Groningen, The Netherlands) system through the working channel of the endoscope.[24–26]

All included patients were sedated during the FMI procedure. First, the endoscope was advanced into the cecum and all bowel segments were inspected with HD-WLE and assessed for inflammation. Second, a fibre bundle (coupled to the custom-built SurgVision Explorer Endoscopy system (SurgVision)) was inserted through the working channel of the endoscope enabling

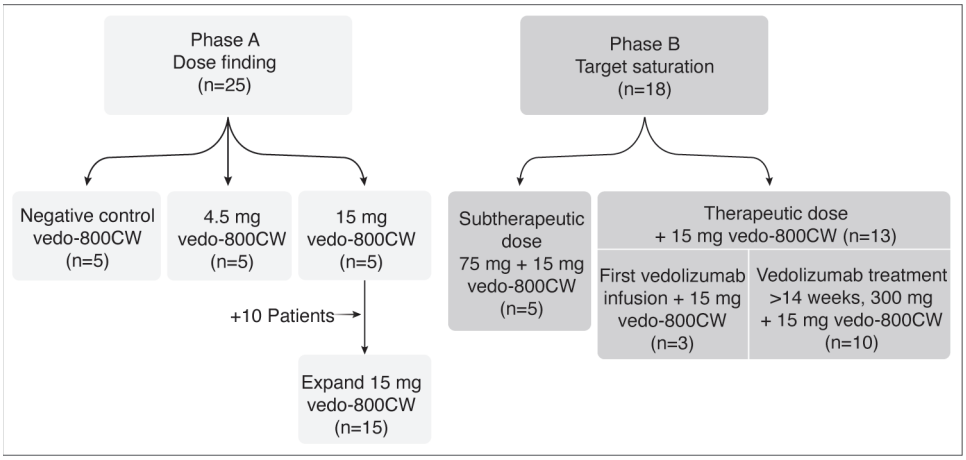

Figure 2 Schematic diagram depicting the various patient cohorts included in phase A and phase B of the study. In total, 43 fluorescence molecular imaging (FMI) procedures were performed. In phase A, three doses of vedo-800CW (0, 4.5 and 15 mg) were administered to vedolizumab-naïve patients (n=5 patients each). The 15 mg cohort was then increased to 15 patients. In phase B, both a subtherapeutic dose (75 mg) and a therapeutic dose (300 or 600 mg) of unlabelled vedolizumab were administered, followed by a 15 mg dose of vedo-800CW. Note that six patients participated in both phase A and phase B; thus, a total of 37 patients were included in this trial.

real-time assessment of the fluorescence signals. Fluorescence images were taken from both active inflamed and non-inflamed tissues in at least two bowel segments. Finally, biopsies were taken from all in vivo FMI-investigated bowel sites and subsequently formalin-fixed paraffin-embedded (FFPE) biopsies. All biopsies were scanned ex vivo using an Odyssey CLx flatbed scanner (LI-COR Biosciences) to generate fluorescence images.

Macroscopic ex vivo imaging and quantification

In each patient, all bowel segments that were assessed in vivo were also assessed ex vivo, constituting of at least one active inflamed segment and one non-inflamed segment. Thus, for all patients at least two segments were included in the analysis, per segment at least two biopsies were taken. All FFPE biopsies were scanned using an Odyssey CLx flatbed scanner (LI-COR Biosciences) to generate fluorescence images. These images were then analysed by drawing a region of interest (ROI) around the complete biopsy using the ImageJ package Fiji,[27] which was used to calculate mean fluorescence intensity (FI_{mean}). The final FI_{mean} was calculated from the results of two independent observers that both drew the ROI around each biopsy to increase its accuracy. Next, FI_{mean} was calculated per bowel segment. Finally, FI_{mean} in each individual patient was calculated by combining all FI values from the separate bowel segments and taking the mean value per category (ie, active inflamed tissue and non-inflamed tissue based on endoscopic assessment of inflammation status). All biopsies were then cut into 4 μm sections for histopathological examination. Specifically, the sections were stained with H&E and used to assess histopathological inflammation severity by an expert GI pathologist who was blinded with respect to the patient cohort.

Microscopic ex vivo imaging

Fluorescence microscopy was used to study the tissue penetration, distribution, accumulation and potential drug–target interactions of vedo-800CW at cellular resolution. For drug and immunofluorescence imaging, select 4 μm FFPE tissue sections were deparaffinised, mounted on glass slides using Prolong Gold Antifade with DAPI mounting medium (Thermo Fisher Scientific), coverslipped and scanned using a Zeiss AxioScan Z1 slide scanner. To investigate the potential interaction between vedo-800CW and specific cell types in the innate and adaptive immune system, the coverslips on previously scanned tissue sections were removed by soaking the slides for 30 min in warm water, and two different fluorescent multiplex immunohistochemistry staining (immune panel 1 and immune panel 2) were performed on serial sections using the Ventana Discovery Ultra Platform (Roche Tissue Diagnostics). A wide range of immune cell types, including adaptive (CD3+ T cells, CD8+ T cells, regulatory T cells, B cells and plasma cells) and innate (dendritic cells, macrophages, eosinophils and neutrophils) immune cells, were chosen as the target cells. Finally, the digital images were analysed using the HALO image analysis platform (Indica Labs, Albuquerque, New Mexico) in order to visualise the tissue distribution of vedo-800CW and to investigate drug–target interactions in specific immune cell subsets. More detailed information on the markers, antibodies used for immunofluorescence and aimed target cells is presented in table S1.

Integrity of the vedo-800CW tracer

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on fresh-frozen patient biopsy samples in order to determine the stability and integrity of the vedo-800CW conjugate. In brief, sample lysates were separated on 7.5% Mini-PROTEAN TGX Precast Gels (Bio-Rad) using a running buffer consisting of Trizma base, glycine and 20% (w/v) SDS at 60 V for 2 hours; two Lonza ProSieve Colour Protein Markers (Fisher Scientific) ranging from 10 kDa to 190 kDa (cat BMA50550) and 4.6–300 kDa (cat BMA00193837) were included as size markers. The gels were scanned using an Odyssey CLx flatbed scanner (LI-COR Biosciences), and fluorescence was visualised at 800 nm.

Statistical analysis

Given the relatively small sample size, all data are considered to be non-normally distributed. Except where indicated otherwise, summary data are presented as the median and IQR. Differences between two groups were analysed using the Mann-Whitney U test (for independent data) or the Wilcoxon signed-rank test (for paired data), and a two-sided p value <0.05 was considered to indicate statistical significance. The data were analysed and plots were generated using Prism V.9 (GraphPad Software, San Diego, California).

RESULTS

A total of 38 patients with IBD were initially included between February 2020 and April 2022. One patient was subsequently excluded from the study because tracer administration was terminated halfway through the infusion due to a Common Terminology Criteria for Adverse Events grade I adverse event (headache); thus, 37 patients were included in the study and final analysis. The patient and disease characteristics of each cohort at baseline are summarised in table 1. Six of the 37 patients in the study participated in both phase A and phase B; thus, in total, 43 FMI procedures were completed.

Macroscopic in vivo and ex vivo imaging and quantification of vedo-800CW

In phase A of this clinical trial, a conventional endoscopic assessment of gut inflammation status showed active inflammation in 32 out of 37 patients. Real-time in vivo macroscopic fluorescence revealed clear uptake of the fluorescent tracer in the target organ—the inflamed gut—compared with healthy (ie, non-inflamed) gut mucosa (figure 3A). Semiquantitative ex vivo FI_{mean} was then used to determine the levels of vedo-800CW fluorescence in both the active inflamed and non-inflamed tissues. FI_{mean} in the active inflamed tissue was significantly higher in the 15 mg dose group in phase A compared with the 4.5 mg dose group, with median (IQR) FI_{mean} values of 153.7 au (132.3–171.4) and 55.3 au (33.6–78.2), respectively ($p=0.0002$). Fluorescence signals were significantly higher in active inflamed tissue compared with non-inflamed tissue in the 15 mg dose group with median (IQR) FI_{mean} values of 153.7 au (132.3–163.7) and 77.7 au (68.8–98.7), respectively ($p=0.0005$), whereas the difference in all other dose groups was non-significant (figure 3B). Negligible signals were found in the control group. Based on this FI_{mean} analysis, 15 mg was chosen for phase B due to the high contrast between inflamed and non-inflamed tissues compared with the 4.5 mg group; this cohort was then expanded to 15 patients by including an additional 10 patients (see figure 2). Furthermore, the 15 mg cohort was analysed based on IBD subtype (UC or CD, with seven and eight patients, respectively) as well as ileocolonic location (left sided, right sided), revealing no significant difference in FI_{mean} between subgroups (figure 3C). In addition, an SDS-PAGE analysis of fresh-frozen biopsy samples obtained

Table 1 Patient characteristics at baseline

	Phase A			Phase B	
Characteristic	**0 mg n=5**	**4.5 mg n=5**	**15 mg n=15**	**75+15 mg n=5**	**Therapy+15 mg n=13**
Age, years	45 (24–53)	42 (30–57)	31 (27–49)	43 (41–57)	46 (37–53)
BMI	26.6 (21.2–28.6)	24.8 (24.6–27.9)	23.4 (21.9–26.9)	29.4 (22.0–33.8)	25.4 (23.0–28.6)
IBD diagnosis					
CD, n (%)	1 (20)	2 (40)	7 (47)	4 (80)	6 (46)
UC, n (%)	4 (80)	3 (60)	8 (53)	1 (20)	7 (54)
Disease activity based on HBI (CD cases) or SCCAI (UC cases)					
Remission, n (%)	1 (20)	2 (40)	4 (27)	2 (40)	2 (15)
Active disease, n (%)	4 (80)	3 (60)	11 (73)	3 (60)	11 (85)
Endoscopic assessment of inflammation status					
Non-inflamed, n (%)	0 (0)	0 (0)	2 (13)	1 (20)	2 (15)
Active inflammation, n (%)	3 (60)	1 (20)	3 (20)	0 (0)	2 (15)
Both non-inflamed and active inflammation, n (%)	2 (40)	4 (80)	10 (67)	4 (80)	9 (69)
Laboratory parameters					
Haemoglobin (mmol/L)	8.4 (7.0–8.4)	8.9 (8.6–9.1)	8.6 (8.1–8.9)	8.0 (7.7–8.4)	7.8 (7.5–8.6)
CRP (mg/L)	7.0 (0.9–16.0)	2.2 (0.5–3.4)	2.6 (1.1–6.0)	16.0 (2.8–16.0)	1.8 (0.6–7.0)
Thrombocytes ($\times 10^9$/L)	329 (273–388)	308 (308–407)	318 (247–363)	297 (286–322)	278 (246–304)
Eosinophils ($\times 10^9$/L)	0.13 (0.07–0.24)	0.07 (0.01–0.13)	0.13 (0.09–0.19)	0.13 (0.09–0.14)	0.12 (0.05–0.23)
Faecal calprotectin	635 (425–3770)	233 (124-473)	605 (295–1710)	160 (100–335)	215 (41–2320)

Except where indicated otherwise, data are presented as the median (IQR).
BMI, body mass index; CD, Crohn's disease; CRP, C reactive protein; HBI, Harvey-Bradshaw Index; SCCAI, Simple Clinical Colitis Activity Index.

from the 15 mg vedo-800CW cohort confirmed the tracer's stability and integrity (online supplemental figure S1).

Next, in phase B we assessed the effect of administering unlabelled vedolizumab on the fluorescent signal. Our ex vivo FI_{mean} analysis revealed a dose-dependent decrease in signal intensity in active inflamed tissues in patients pretreated with increasing doses of unlabelled vedolizumab followed by vedo-800CW. Specifically, patients who received a subtherapeutic dose (75 mg) followed by 15 mg vedo-800CW had a median (IQR) FI_{mean} of 101.6 au (85.7–163.7). Patients who received their first therapeutic dose followed by 15 mg vedo-800CW had an FI_{mean} of 69.2 au (60.2–82.4), and patients who received their therapeutic dose after >14 weeks of therapy followed by 15 mg vedo-800CW had a median FI_{mean} of 59.3 au (50.1–90.7). Compared with the vedolizumab-naïve patients in the 15 mg vedo-800CW cohort in phase A, FI_{mean} was significantly reduced by more than 61% ($p<0.0001$) in the therapeutic dose cohort in phase B (figure 3A,B). Moreover, FI_{mean} in the therapeutic dose cohort was decreased to the same level measured in non-inflamed tissues in these patients, suggesting that vedo-800CW binding was blocked by unlabelled vedolizumab in the inflamed mucosa.

Microscopic analysis of drug distribution and drug–target interaction

Next, we performed ex vivo fluorescence microscopy in order to assess the distribution of vedo-800CW in both 20, 10 and 4 μm tissue sections (online supplemental figure S2). Our analysis revealed deep penetration and a heterogeneous distribution of vedo-800CW in affected tissue samples; specifically, the tracer was not located exclusively inside the vessels but also migrated into the gut mucosa. Furthermore, our microscopic analysis confirmed our previous macroscopic findings in which FI was higher in the 15 mg vedo-800CW phase A cohort compared with the therapeutic dose+15 mg vedo-800CW cohort in phase B. The control group showed only autofluorescent signals (figure 4A).

To study the drug–target interaction and identify the cell types targeted by vedo-800CW, we performed a more detailed analysis of vedo-800CW binding to a variety of immune cell types ranging from surface binding to internalisation of the drug (indicating cytoplasmatic uptake) (figure 4B). We observed surface binding between vedo-800CW and plasma cells, as well as intracellular localisation of vedo-800CW in both eosinophils and macrophages (figure 4C). Though we did not find clear vedo-800CW positive T cells, CD3+ and CD8+ cells were abundant in regions with strong vedo-800CW signal. Finally, we found no clear evidence of binding between vedo-800CW and any of the other immune cell types studied.

DISCUSSION

To the best of our knowledge, this is the first study designed to visualise the macroscopic and microscopic distribution of an intravenously administered fluorescent form of vedolizumab in the gut mucosa. Using FMI, we found a dose-dependent increase in vedo-800CW fluorescence in actively inflamed tissues, demonstrating that this approach can be used to assess mucosal drug distribution both in vivo and ex vivo. We also found that the delivery of unlabelled vedolizumab prior to vedo-800CW administration reduced vedo-800CW fluorescence to the level measured in non-inflamed tissues, indicating specific targeting and suggesting saturation of the target tissue. Furthermore, we found that vedolizumab targets a variety of immune cell types in the inflamed mucosa. Together, these findings represent the first step towards better understanding of the distribution of vedolizumab in the inflamed gut mucosa, thereby helping future research to design studies to investigate the drug's mechanism of action.

The ability to assess the macroscopic distribution of a drug can be important for confirming that the drug reaches its target site. Moreover, it may also help provide new insights in the drug's working mechanism and may eventually help predict the patient's response to therapy. Molecular imaging is considered

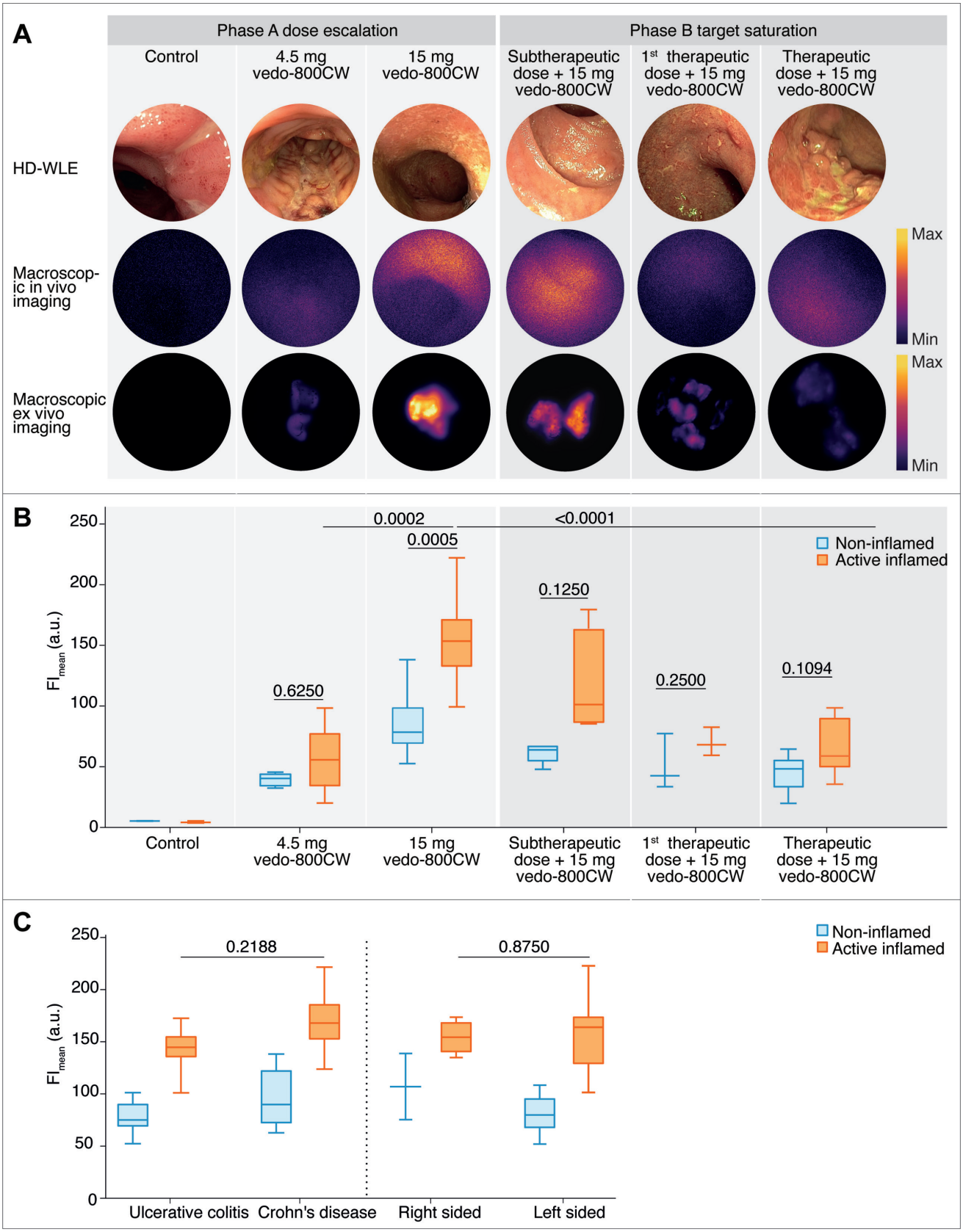

Figure 3 In vivo visualisation and ex vivo quantification. (A) Representative high-definition white light endoscopy (HD-WLE) images (top row) of active inflamed tissue, with corresponding in vivo (middle row) and ex vivo (bottom row) fluorescence images in the indicated cohorts. All fluorescence images were scaled per modality in relation to one another to allow interimage comparisons. (B) Box plot summarising mean fluorescence intensity (FI_{mean}) calculated from fluorescence scans of all biopsies in all cohorts, with p values indicated (Mann-Whitney U test). (C) Box plot summarising the results obtained from the 15 mg cohort in phase A, stratified by disease subtype and disease location, with p values indicated (Mann-Whitney U test).

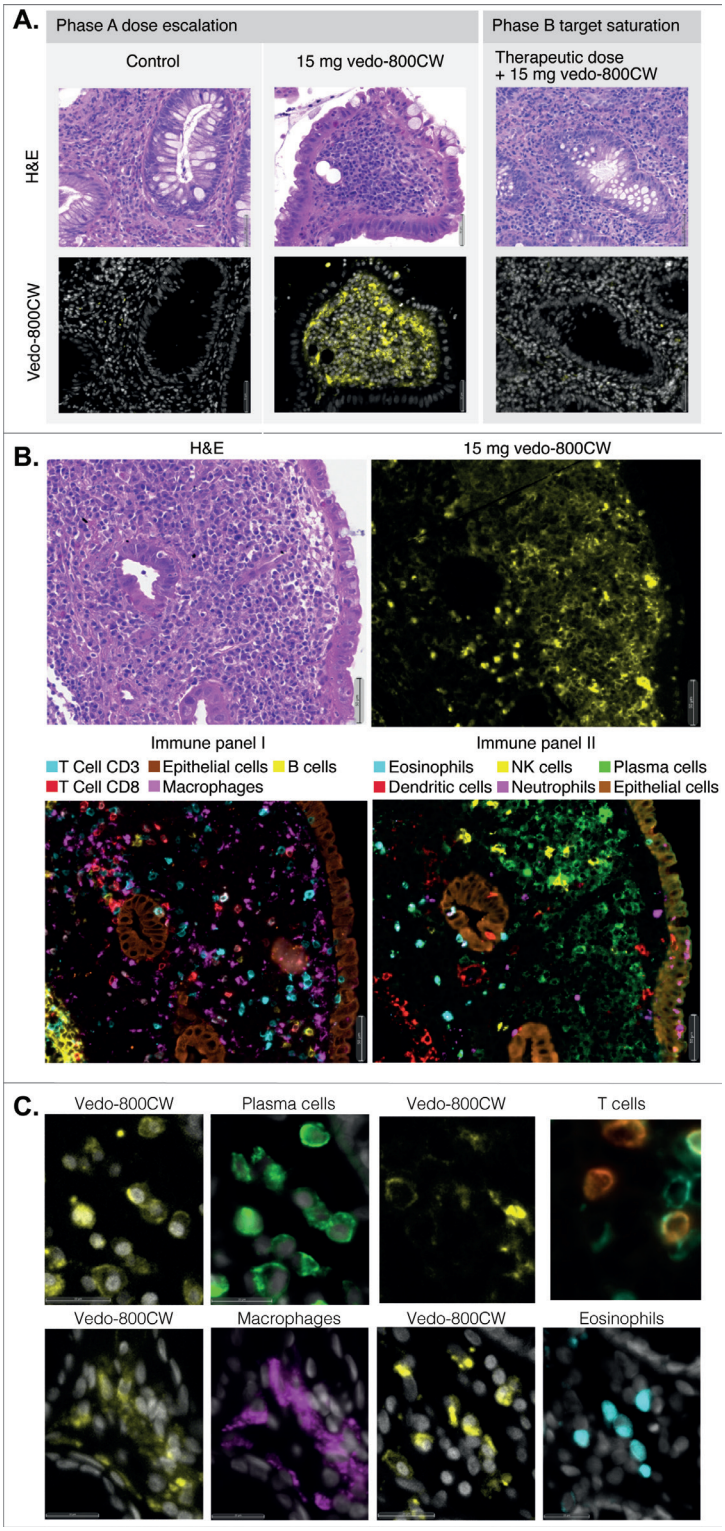

Figure 4 Microscopic distribution of vedo-800CW and target cell identification. (A) Representative fluorescence microscopy images of biopsy sections obtained from patients with active inflammation in the 0 mg cohort (left), the 15 mg cohort (middle) and the therapy+15 mg cohort (right); the nuclei were counterstained with DAPI shown in grey. Shown above each vedo-800CW fluorescence image is the corresponding H&E-stained section. Note the considerably stronger fluorescence signal in the patient in the 15 mg group compared with the other two patients. (B) Representative images showing an active inflamed H&E section (left) with the adjacent correlation between the distribution of vedolizumab (measured as vedo-800CW fluorescence; right) in tissue sections. The same sections immunostained for two distinct immune panels are shown below. Note that the section stained for immune panel II (right) shows an abundance of plasma cells (green) in an area also containing a high number of vedo-800CW-positive cells (yellow), while the section stained for immune panel I (left) shows colocalisation between vedo-800CW fluorescence and macrophages (purple). In contrast, there is little correlation between vedolizumab localisation and CD3+ or CD8+ T cells (stained green in the red and blue). (C) Representative high-magnification images showing the spatial correlation between either surface or intracellular vedo-800CW fluorescence (left images in each pair) and specific immune cell types (right images). NK, natural killer.

a powerful tool for increasing our understanding of a drug's distribution and predicting therapeutic outcome; however, to date these studies primarily involved positron emission tomography and were performed in an oncology setting.[28 29] With respect to IBD, Atreya *et al* previously reported a study in which they sprayed Fluorescein isothiocyanate (FITC)-labelled adalimumab during endoscopy and then used confocal laser endomicroscopy (CLE) to identify tumour necrosis factor-alpha-expressing cells.[30] Furthermore, Rath *et al* reported the first use of FITC-labelled vedolizumab in IBD. In their pilot study, the authors visualised α4β7-expressing cells in two patients with CD using fluorescence microscopy and ex vivo CLE. Both patients responded to vedolizumab therapy, whereas three other patients with CD who did not respond to treatment had no α4β7-expressing cells.[31] These findings suggest that molecular imaging may be used together with fluorescently labelled vedolizumab as a predictive tool. However, the authors' use of topical application in both studies is an in vivo immunohistochemistry method and does not necessarily reflect the drug's tissue distribution during therapy. In contrast, we delivered fluorescently labelled vedolizumab systemically via intravenous injection, thus providing visual information regarding its in vivo and ex vivo distribution in the inflamed mucosa. In addition, we evaluated the in vivo target saturation during vedolizumab therapy and obtained new insights regarding vedolizumab's target cells, thus making important steps towards understanding of vedolizumab treatment in IBD. Similarly, the ability to quantify local drug levels may be useful in cases in which plasma levels do not reflect the concentration at the site of action. For instance, studies have shown that only ∼5% of a systemically administered drug reaches the inflammatory site or tumour.[32] Moreover, a clinical study has shown that vedolizumab concentrations in the mucosa are significantly lower compared with vedolizumab levels in serum (10.54 and 23.02 µg/mL, respectively), but are associated with objective response rates, whereas vedolizumab serum levels were deemed unsuitable for therapeutic drug monitoring.[33] In this respect, in vivo FMI can be used to qualitatively assess areas with relatively high or low FI, while semi-quantification in biopsies can be used to measure FIs ex vivo. Moreover, our finding that vedo-800CW remains intact based on SDS-PAGE analysis of fresh-frozen biopsy samples indicates that high fluorescence levels likely correspond to high local drug levels. Our dose-finding study revealed that 15 mg vedo-800CW provided a stronger signal than 4.5 mg and showed a significant difference in fluorescence between actively inflamed tissues and non-inflamed tissues. Interestingly, we also found that binding of vedo-800CW in inflamed tissue was significantly reduced even by a subtherapeutic (75 mg) predose of unlabelled vedolizumab, and this blocking effect was increased further in patients receiving a therapeutic dose of unlabelled vedolizumab. In addition, our ex vivo macroscopic fluorescence measurements revealed that the vedo-800CW signal was reduced to the same extent both in patients who received their first therapeutic dose of unlabelled vedolizumab and in patients who previously received more than 14 weeks of therapy. Hence, it is reasonable to speculate that vedolizumab saturates the target tissue even after only a single therapeutic dose. This hypothesis was underscored by our fluorescence microscopy results in patients on vedolizumab treatment, where no specific binding of vedo-800CW to plasma cells was observed. This implies that virtually all α4β7 target molecules were bound by non-fluorescent vedolizumab.

To gain additional insights into a drug's mechanism of action, it is essential to visualise its microscopic distribution and identify its target cells. With respect to vedolizumab, whether cells other than T cells play a role in its mechanism of action remains an open question.[14 19] Here, we show that vedo-800CW is distributed in a dose-dependent manner in the inflamed mucosa, and it binds to and/or is taken up by a variety of immune cell types. Specifically, our fluorescence microscopic analysis revealed both surface and intracellular vedo-800CW binding. In addition, consistent with recent findings,[14 17 19] we found that vedolizumab binds to a variety of immune cell types, including plasma cells, macrophages and eosinophils, whereas an actual correlation between T cells and vedo-800CW was not observed. These findings support the hypothesis that migration of α4β7-expressing T cells to the mucosa is prevented and that these T cells are likely not the sole therapeutic target of vedolizumab. Furthermore, it was shown that the binding of vedolizumab to α4β7 leads to internalisation of the antibody-integrin complex in peripheral blood T cells in vitro.[9] In vivo, this internalisation likely further contributes to the inhibition of T cell migration by preventing the binding of α4β7 to MadCAM.

The potential effect of vedolizumab on plasma cells was described previously. For example, in 1995 Farstad *et al* reported an abundance of α4β7-expressing plasma cells in the lamina propria.[34] Moreover, Canales-Herrerias *et al* recently reported in a preprint that vedolizumab can affect the abundance of mucosal plasma cells.[10 19] Nevertheless, vedolizumab was shown to have opposite effects on the abundance of specific plasma cell subtypes, increasing some subtypes but decreasing others.[10 19] Here, we found intracellular localisation of vedolizumab in both macrophages and eosinophils, consistent with previous studies showing an interaction between vedolizumab and α4β7-expressing macrophages and eosinophils.[12 17 35] Although this interaction between vedolizumab and various immune cell types is established, the consequences of this drug binding to different immune cell types remain poorly understood and require further study. Previous studies demonstrated that α4β7 receptors are saturated at very low vedolizumab doses,[36] whereas we show that a higher dose of vedolizumab is necessary to achieve full target saturation at the inflamed mucosa. We believe this discrepancy may originate from the fact that solely saturation of the α4β7 receptors on T cells in serum was investigated, whereas the potential role of vedolizumab on other immune cells in the inflamed mucosa was not included in these studies.

Our study has several limitations that warrant discussion. First, due to the relatively low number of vedolizumab-naïve patients and patients enrolled after 14 weeks of vedolizumab therapy, we are unable to draw any conclusions regarding whether FMI can predict the patient's response to therapy. Ideally, we would have included a group receiving 300 mg vedo-800CW. However, due to production limits of the compound in this phase I feasibility study this was not yet feasible. Moreover, we were unable to compare between patients with CD and patients with UC and between several bowel segments. In addition to differences between patients, we also observed heterogeneity within individual patients, even in a single biopsy. This raises the question of what ultimately drives vedolizumab's biological effect. To address this important question, future studies should include larger cohorts of patients with UC and CD who undergo vedo-800CW FMI at baseline and following their therapy. Additional analyses conducted at the immunological priming sites in which α4β7 expression is induced in immune cells, or in peripheral blood immune cells, may provide new information for predicting treatment response. Given the complexity of these diseases we also believe that combining our approach with more in-depth techniques such as spatial transcriptomics, single cell and RNA sequencing may be needed in order to predict response on

vedolizumab treatment and determine the precise mechanism of action. A second limitation was that our automated quantification of FI during fluorescence microscopy was hampered by the presence of autofluorescence. However, the ability to perform in vivo quantification during endoscopy may increase our ability to predict response, and this technique is currently being developed in an ongoing EIC Horizon Pathfinder project (grant number 101046923).

Our approach using FMI to visualise fluorescently labelled drugs in inflammatory diseases can also be used to gain insights into drug distribution and identify target cells in other contexts. For example, our group is currently using FMI to visualise fluorescently labelled adalimumab and ustekinumab in arthritis, psoriasis and IBD (ClinicalTrials.gov trials NCT03938701 and NCT05725876). Using a similar approach may also be valuable in early drug development trials. Measuring the local drug concentration, identifying the drug's target cells and determining target saturation can provide important information for making go/no-go decisions during development.[37] As a result, the drug development process is expedited, costs are reduced and patient selection accuracy is improved upon the introduction of new drugs to the market.

In conclusion, this phase I feasibility study using a novel optical imaging approach provides the first detailed information regarding the macroscopic and microscopic distribution of vedolizumab in the inflamed gut, including information regarding its target cells. Our stepwise FMI approach including in vivo fluorescence endoscopy, ex vivo fluorescence analysis of biopsies and fluorescence microscopy has the potential to increase the understanding of the underlying mechanism of action, the drug's distribution and its targets, leading to the optimisation of treatment in individual patients.

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