A Novel Tumor-Specific Agent for Intraoperative Near-Infrared Fluorescence Imaging: A Translational Study in Healthy Volunteers and Patients with Ovarian Cancer

Charlotte E.S. Hoogstins, Quirijn R.J.G. Tummers, Katja N. Gaarenstroom, Cor D. de Kroon, J. Baptist M.Z. Trimbos, Tjalling Bosse, Vincent T.H.B.M. Smit, Jaap Vuyk, Cornelis J.H. van de Velde, Adam F. Cohen, Philip S. Low, Jacobus Burggraaf, and Alexander L. Vahrmeijer

Abstract

**Purpose:** Completeness of cytoreductive surgery is a key prognostic factor for survival in patients with ovarian cancer. The ability to differentiate clearly between malignant and healthy tissue is essential for achieving complete cytoreduction. Using current approaches, this differentiation is often difficult and can lead to incomplete tumor removal. Near-infrared fluorescence imaging has the potential to improve the detection of malignant tissue during surgery, significantly improving outcome. Here, we report the use of OTL38, a near-infrared (796 nm) fluorescent agent, that binds folate receptor alpha, which is expressed in >90% of epithelial ovarian cancers.

**Experimental Design:** We first performed a randomized, placebo-controlled study in 30 healthy volunteers. Four single increasing doses of OTL38 were delivered intravenously. At fixed times following drug delivery, tolerability and blood/skin pharmacokinetics were assessed. Next, using the results of the first study, three doses were selected and administered to 12 patients who had epithelial ovarian cancer and were scheduled for cytoreductive surgery. We measured tolerability and blood pharmacokinetics, as well as the ability to detect the tumor using intraoperative fluorescence imaging.

**Results:** Intravenous infusion of OTL38 in 30 healthy volunteers yielded an optimal dosage range and time window for intraoperative imaging. In 12 patients with ovarian cancer, OTL38 accumulated in folate receptor alpha–positive tumors and metastases, enabling the surgeon to resect an additional 29% of malignant lesions that were not identified previously using inspection and/or palpation.

**Conclusions:** This study demonstrates that performing real-time intraoperative near-infrared fluorescence imaging using a tumor-specific agent is feasible and potentially clinically beneficial.

Introduction

The completeness of surgical tumor removal is an important factor for determining the survival of patients with a solid tumor. Despite advances in preoperative imaging techniques, during surgery, the surgical oncologist must rely primarily upon inspection and/or palpation to identify the tumor tissue; however, these methods are often inadequate (1–3).

Ovarian cancer has the highest mortality rate of all gynecologic cancers (4). The surgical treatment of advanced-stage ovarian cancer (i.e., International Federation of Gynecology and Obstetrics stage IIb to stage IV) typically consists of cytoreductive surgery combined with systemic chemotherapy. Several studies have shown that the amount of residual tumor that remains following cytoreductive surgery is the most important prognostic indicator of survival (5–9). Thus, because imaging modalities that improve tumor identification during surgery can increase the number and thoroughness of metastatic lesions resected during cytoreductive surgery, they can significantly improve patient outcome.

Near-infrared (NIR) fluorescence imaging is an innovative technique that can be used to detect tumor lesions during surgery (10). NIR fluorescence is invisible to the human eye, but can be detected in the millisecond range using a dedicated imaging system. Because the imaging system can be toggled on and off rapidly, this approach allows the surgeon to identify malignant tissue in real time without altering the surgical field. In addition, NIR light can penetrate tissue in the order of centimeters, allowing the surgeon to delineate targets underneath the tissue surface.
Translational Relevance

Here, we report the clinical translation of a tumor-specific near-infrared fluorescent agent for intraoperative imaging during surgery for ovarian cancer. Our unique approach using a mixed population of healthy subjects and patients in the same phase I protocol allowed us to rapidly determine the optimum dose and time window for performing intraoperative imaging. Using an optimized imaging system, a fluorescent signal was visible in the primary tumor and/or metastases of all patients, facilitating the resection of an additional 29% of lesions that were not observed by inspection and/or palpation. The completeness of cytoreductive surgery is a key prognostic factor for survival of patients with ovarian cancer. By visualizing the tumor in real time using this novel intraoperative imaging approach, the surgical outcome of patients with ovarian cancer can be improved considerably. As more agents become available, this approach could herald a paradigm shift within the entire field of surgical oncology.

Despite its high potential in clinical applications, NIR fluorescence in surgical oncology has been used primarily with nonspecific agents previously available for clinical use. For example, indocyanine green is retained either in or around tumor tissue due to impaired secretion or increased vascular permeability and decreased lymphatic drainage; however, indocyanine green does not bind specifically to cancer tissue (13, 14). This lack of specificity results in a high rate of intraoperative false positive images in patients with ovarian cancer (15). Thus, fluorescent agents that specifically target cancer-specific targets are highly desired.

Folate receptor alpha (FRα) is a promising target, as it is robustly expressed on a variety of cancers of epithelial origin, including >90% of epithelial ovarian cancers (16–18). Moreover, FRα is expressed at relatively low levels in healthy tissue, where it is expressed primarily at the apical membrane of polarized epithelial cells, including fallopian tube and endometrial tissue (16, 19, 20). Thus, when targeted with a fluorescent agent, background fluorescence will be low in healthy tissue, making this protein an ideal candidate target for fluorescence-guided ovarian cancer surgery. Importantly, because chemotherapy does not affect the expression of FRα, this protein can be targeted in both primary and interval cytoreductive surgical procedures (21, 22).

The potential of using a folate analogue coupled to a dye that fluoresces outside of the NIR spectrum (e.g., folate-FITC) was demonstrated previously in a small patient series, yielding a positive fluorescence signal in 3 of 4 patients with ovarian cancer (23). Although this study showed the feasibility of detecting fluorescently labeled tumor deposits in real time, the approach did not allow the surgeon to detect lesions beneath the tissue surface. In addition, we found that using folate-FITC in both ovarian cancer and breast cancer produces high autofluorescence in healthy tissue (data not shown). This finding underscores the need for agents that fluoresce in the NIR spectrum.

OTL38 is a folate analogue conjugated to a NIR fluorescent dye (excitation at 776 nm, emission at 796 nm); OTL38 has high specificity and affinity for FRα. Here, we first examined the tolerability, pharmacokinetics, and tissue and blood distribution of increasing doses of OTL38 in healthy volunteers. Based upon these results, we then determined the optimal dosage range and the imaging time window. We then used these parameters in a study in patients with epithelial ovarian cancer to determine the correlation between fluorescence detection and histopathology of the resected lesions. We also determined whether the detection of tumors using the traditional surgical view was improved with the addition of fluorescence imaging.

Materials and Methods

Study design

The primary objective of the study in healthy volunteers was to assess the tolerability and pharmacokinetics (in plasma and skin) of single intravenous doses of OTL38; in addition, the results were used to determine the optimal dosage range and time window for performing intraoperative imaging in the subsequent study in patients with ovarian cancer. For the patient study, the objectives were to assess the tolerability and pharmacokinetics of OTL38, the efficacy with respect to intraoperative detection of ovarian cancer lesions, and the practical feasibility of the technique. The results were used to determine the optimal dose for intraoperative imaging. Because both studies were exploratory in nature, sample size was not based on a formal calculation of statistical power. In the first study, tolerability and pharmacokinetics of a single intravenous dose of OTL38 were used as the endpoints. These same endpoints were used in the patient study; in addition, we also measured the efficacy of OTL38 in the intraoperative detection of ovarian cancer by measuring the following endpoints: tumor-to-background ratio (TBR), defined as the ratio between the fluorescent signal in the tumor tissue and the fluorescent signal in the tissue surrounding the tumor; concordance between the pathology results with respect to the presence of cancer and the imaging assessment; the number and location of FRα-positive, cancerous lesions identified the usual visual, and/or tactile approaches with or without fluorescence imaging; and the surgeons’ evaluation of the practical application of the technique. Data of all subjects participating in the studies were included in the analyses if the data could meaningfully contribute to the objectives of the studies.

For the study in healthy volunteers, we included 30 subjects who were 18 to 65 years of age and were considered healthy based on medical screening. For the patient study, we included 12 patients who had a high suspicion of epithelial ovarian cancer or a tissue-based diagnosis of epithelial ovarian cancer and were scheduled for primary or interval cytoreductive surgery. The main exclusion criteria were current pregnancy, history of anaphylactic reactions, impaired renal function (defined as eGFR <50 mL/min/1.73 m²), and impaired liver function (defined as alanine aminotransferase, aspartate aminotransferase, or total bilirubin levels that exceeded three times the established upper limit of normal).

The study in healthy volunteers was a randomized, placebo-controlled design in which subjects were randomized to receive a single intravenous dose of 0.025, 0.05, 0.1, or 0.2 mg/kg OTL38 or placebo. The randomization code was generated by a study-independent statistician using SAS 9.1.3 for Windows (SAS Institute Inc.). The randomization code was made available for data analysis only after the study was completed. At fixed time points following administration, blood samples were collected and used to measure pharmacokinetics and to perform routine laboratory tests. Adverse events, ECG, and vital signs were recorded. The
fluorescent signal in superficial skin was measured using the Artemis imaging system at fixed time points following intravenous administration of OTL38 or placebo (Fig. 1). After a dose cohort was completed, all data collected up to 24 hours following each dose were reviewed prior to increasing the dose. In the event of an unacceptable tolerability profile (based on the nature, frequency, and intensity of adverse events, as judged by the investigator), the dose was not increased. Subjects were assigned to a dosing group based on the order in which they enrolled in the study. The study was performed in a double-blind fashion; thus, the investigator, staff, subjects, sponsor, and monitor were blinded with respect to the treatment until the end of the study. The placebo and OTL38 were formulated and packaged identically. The randomization list was made available only to the pharmacist who prepared the study drug, the individual who was responsible for sample bioanalysis, and the statisticians and programmers who prepared the blinded summaries, graphs, and listings to support the dosing decisions.

The patient study was a single ascending dose, open-label exploratory study. The patient study was not randomized, and all patients received the active drug. Assignment to the dosage groups was based on the order in which the patients enrolled in the study. The patients received a 1-hour intravenous infusion of OTL38 2 to 3 hours prior to the start of surgery. A dose-escalating scheme with planned doses of 0.025, 0.05, and 0.1 mg/kg (and the possibility to decrease the dose to 0.0125 mg/kg) was used. Dose escalation was terminated in the event of an unacceptable tolerability profile. Tolerability assessment (blood pressure, pulse, peripheral oxygen saturation, respiratory rate, ECG, temperature, and skin assessments) and blood collection for pharmacokinetics and routine laboratory tests were performed at regular intervals starting just prior to administration and lasting until 24 hours after dosing. Adverse events and the concomitant use of other medications were recorded. Cytoreductive surgery generally included the removal of the uterine adnexa, uterus, and infracolic omentum, as well as resection of all macroscopic tumors, where possible. All surgical procedures were open procedures performed by an experienced gynecologic oncologist using a midline abdominal incision. First, the primary tumor and metastases were identified in the surgical field using standard visual and tactile methods. Thereafter, the Artemis imaging system was used to identify NIR-fluorescent lesions. All tumor tissue identified by visual/tactile methods and/or NIR fluorescence was resected, provided it was both surgically feasible and clinically useful. Each resected lesion was marked on a case report form as being either fluorescent or nonfluorescent and as being either clinically suspected of malignancy or not (Supplementary Fig. S1). All resected lesions were examined for tumor status by an experienced pathologist. A positive tumor that was fluorescent was considered a true positive; a negative lesion that was
fluorescent was considered a false positive; and a positive tumor that was nonfluorescent was considered a false negative. In addition, we performed IHC to demonstrate FRα and FRβ expression coupled with fluorescence microscopy in order to evaluate OTL38 binding (Supplementary Materials and Methods).

Investigational product
OTL38 (chemical formula: C₆₁H₆₃N₉Na₄O₁₇S₄; molecular weight: 1414.42 Da) consists of a folate analogue conjugated to an NIR fluorescent dye. OTL38 (>96% purity) was obtained from On Target Laboratories (West Lafayette). The drug was synthesized and manufactured at Aptuit in compliance with Good Manufacturing Practices (Supplementary Fig. S2). OTL38 was stored in frozen form at −20°C in vials containing 6 mg OTL38 free acid in 3 mL water. Before administration, the frozen vials were thawed, vortexed, and then diluted with 0.9% NaCl or 5% dextrose for intravenous infusion. OTL38 was diluted in either 20 or 220 mL and was infused over 10 or 60 minutes. Placebo consisted of a similar volume of 0.9% NaCl or 5% dextrose.

Intraoperative NIR fluorescence imaging system
Imaging was performed using the Artemis fluorescence imaging system (Quest Medical Imaging; ref. 24). The system consists of three wavelength-isolated light sources, including a ‘white’ light source and two separate NIR light sources. For this study, the camera and light engine where optimized for use with OTL38; specifically, they were designed to generate 7.5 mW/cm² at 760-nm light. Color video and fluorescence images were acquired simultaneously using separate sensors and were displayed in real time using custom-built optics and software, thereby displaying color video and NIR fluorescence images separately. A pseudo-colored (lime green) merged image of the color video and fluorescence images was also generated. The intensity of the light source was controlled using the Artemis software. The camera was attached to a freely moveable arm. During surgery, the camera and moveable arm were enclosed in a sterile shield and drape (Medical Technique Inc.).

Ethics committee approval
Both studies were performed in accordance with the tenets established by the Helsinki Declaration of 1975 (as amended in Tokyo, Venice, Hong Kong, Somerset West, Edinburgh, Washington, and Seoul), ICH-GCP guidelines, and the laws and regulations of the Netherlands. In addition, both studies were approved by a certified medical ethics review board. All subjects provided written informed consent prior to the start of any study-related procedures. The healthy volunteer study and ovarian cancer patient study were registered in the European Clinical Trials Database under numbers 2013-004774-10 and 2014-002352-12, respectively; publicly accessible via the CCMO register (https://www.toetsingonline.nl/to/ccmo_search.nsf/Searchform?OpenForm).

Practical evaluation
Directly following the surgical procedure, the surgeon was asked to complete a questionnaire regarding the practical application of the technique during the surgical procedure (Supplementary Materials and Methods).

Visual detection
Color and fluorescence images of seven representative surgical views of patients with confirmed ovarian cancer were captured from the videos recorded using the Artemis imaging system. Intraobserver variability was assessed by including a matching color and fluorescence image set twice (one set was a horizontal mirror image of the original), resulting in a total of eight sets of matching color and fluorescence images that were printed in full color; representative images are shown in Fig. 2. Three experienced gynecologic oncologists were asked to mark clinically suspected lesions directly on the color images; they were then asked to mark clinically suspected lesions on the matching fluorescence images. Visual detection was performed ex vivo because intraoperative assessment of the number of lesions was not feasible.

Pharmacokinetics analysis
The bioanalysis was performed using validated methodologies in compliance with good clinical laboratory practices at Analytical Biochemical Laboratory. In brief, OTL38 was extracted from human K2EDTA plasma samples and urine samples using offline solid-phase extraction, followed by analysis using liquid chromatography/mass spectrometry (APL-4000; Attodyne Inc.). The assay’s lower limit of quantification (LLOQ) and upper limit

![Figure 2](Image)

**Figure 2.** Visual detection of tumor deposits ex vivo. A, representative color (top) and fluorescence (bottom) images used to quantify the visual detection of tumor deposits. B, box plot summarizing the number of tumor lesions detected based on the matched color and fluorescence images.
of quantification (LLOQ) were 2.00 and 500 ng/mL, respectively. The coefficient of variability for intra-day and inter-day plasma LLOQ and urine LLOQ was 8.2% and 13.3%, respectively.

Statistical analysis
SPSS statistical software package (version 20.0, IBM Corp.) was used for statistical analyses. The individual OTL38 concentration–time profiles were analyzed using noncompartmental methods. The obtained pharmacokinetic parameters (i.e., AUC, C_max and t_max) were summarized per treatment group, including the number of subjects, mean values, SD, median values, and minimum and maximum values. The fluorescence signal in the skin (healthy volunteers) or tumor and background tissue (patients) was quantified using ImageJ (version 1.49b, NIH, Bethesda, MD; http://image.nih.gov/ij/). Using ImageJ, a region of interest (ROI) was drawn on the images and used to quantify the fluorescence signal in arbitrary units (AU). TBR was calculated by dividing the fluorescence signal of the tumor by the fluorescence signal of the surrounding tissue. To compare the TBR values and fluorescence background signals between malignant and benign (i.e., false positive) lesions and between different dose groups, an independent samples Student t test was performed. TBR is reported as the mean, SD, and range. Patient characteristics are reported as the median, SD, and range. Tumor deposits that were marked on the color and fluorescence images were counted. To compare the number of deposits between the color and fluorescence images, a paired Student t test was performed.

Results
Clinical trial in healthy volunteers
This study included a total of 30 subjects (18 females and 12 males) 18 to 64 years of age with a BMI of 18 to 30 kg/m² (see Supplementary Fig. S3 for the CONSORT flow diagram).

Tolerability
OTL38 at 0.025 mg/kg diluted in 20 mL 0.9% NaCl infused for 10 or 60 minutes caused moderate hypersensitivity in two of the four subjects receiving this dose. These reactions were not classic allergic reactions, as they were not accompanied by an increase in tryptase or IgE, nor did they involve the complement system (see Supplementary Data File S1). Subsequent studies using dynamic light scattering and scanning electron microscopy revealed that these reactions might have been due to aggregation of the OTL38 compound in the 0.9% NaCl solution. This aggregation was reduced considerably when OTL38 was diluted in 5% dextrose and when the infusion volume was increased. Because OTL38 did not aggregate measurably when diluted to 7.5 μmol/L in 5% dextrose, the study was restarted at the lowest dose with OTL38 dissolved in 220 mL 5% dextrose; this volume was infused for a period of 60 minutes.

Infusion of 0.025, 0.05, and 0.1 mg/kg OTL38 diluted in 5% dextrose was associated with mild adverse events that disappeared gradually during and/or after the infusion. These adverse events were dose dependent and suggestive of hypersensitivity (e.g., abdominal discomfort, nausea, and pruritus), but did not require intervention. All adverse events are listed in Supplementary Table S1. At the 0.2 mg/kg dose, some subjects developed adverse events of moderate severity, which required the temporary interruption of the infusion or the administration of an antihistamine (e.g., 1 to 2 mg clemastine intravenously). Overall, more than 80% of these adverse events were mild in severity, and all other adverse events were moderate in severity. Despite the development of adverse events, the infusion of OTL38 at 0.025 to 0.2 mg/kg did not cause clinically meaningful changes relative to baseline with respect to laboratory values, ECG, or vital signs.

Pharmacokinetics
The maximum blood plasma concentration was achieved with each dose immediately at the end of the infusion and declined thereafter with a half-life of 2 to 3 hours (Supplementary Fig. S4). Supplementary Table S2 summarizes the most important pharmacokinetic parameters in each treatment group.

OTL38 excreted in the urine (expressed as a percentage of the dose administered) increased with increasing dose, and the highest level was approximately 11% for the highest dose (0.2 mg/kg). It is, therefore, reasonable to assume that the relatively low recovery is due in part to the lower limit of detection for OTL38 in urine.

Pharmacokinetics in superficial tissue
Figure 1 shows example images obtained using the Artemis imaging system. Although OTL38 was cleared from the plasma 2 to 3 hours after intravenous infusion, our analysis of the fluorescence signal in the skin revealed that fluorescence increased initially, remained increased for 6 hours, and then decreased with a half-life of approximately 15 hours (Fig. 1).

Clinical trial in patients with ovarian cancer
Fourteen patients initially enrolled in this study. However, because the study drug was temporary unavailable, one patient could not participate, and another patient withdrew from the study. Thus, a total of 12 patients, 49 to 77 years of age with a BMI of 20 to 41 kg/m² received OTL38 (see Supplementary Fig. S5 for the CONSORT flow diagram). The surgical procedure, histology, differentiation grade, and International Federation of Gynecology and Obstetrics (FIGO) stages are summarized in Table 1.

Dose escalation
Patients 1, 2, and 3 received a starting dose of 0.025 mg/kg. After we reviewed the safety and efficacy data, the dose was increased to 0.05 mg/kg in the next three patients. However, when the three patients 4, 5, and 6 received this higher dose, the number and severity of symptoms (primarily consisting of abdominal discomfort, nausea, and pruritus) increased. In addition, TBR appeared to decrease. Therefore, the dose was decreased to 0.0125 mg/kg for the next three patients (patients 7, 8, and 9), yielding fewer, less severe symptoms, and an increase in TBR. Nevertheless, even at this lowest dose, mild, self-limiting adverse events were reported. After reviewing the safety and efficacy data collected using all three doses, 0.0125 mg/kg was chosen as the optimal dose for the expansion cohort (patients 10, 11, and 12).

In retrospect, this lower dose was a good choice, as adverse symptoms were minimal and TBR was maximal. Supplementary Table S3 and Supplementary Fig. S6 summarize the TBR results.

Other adverse events
One patient who received a dose of 0.0125 mg/kg OTL38 developed a case of postoperative hospital-acquired pneumonia and coughing-induced wound dehiscence. These complications were considered unrelated to OTL38 administration. The complete list of all adverse events recorded in the patients is provided...
Administration of OTL38 itself did not lead to any obvious changes in laboratory values, ECG, vital signs, or temperature.

**Pharmacokinetics**

The pharmacokinetic profile of OTL38 in patient blood was similar to the profile measured in the healthy volunteers. Specifically, with each dose, the maximum concentration was achieved at the end of the infusion. After stopping the infusion, plasma concentration decreased with a half-life of 2 to 3 hours (Supplementary Fig. S4).

**Intraoperative NIR fluorescence imaging**

Lesions could be detected clearly after OTL38 administration. The optimal camera exposure time was dependent on OTL38 dose, with lower doses requiring longer exposure times. At higher doses, the longer exposure time led to saturated images; however, in all cases, it was possible to use a sufficiently brief exposure time to obtain real-time images. Figure 3 shows an example of fluorescent lesions that were subsequently confirmed as ovarian cancer metastases on histopathology. A total of 83 fluorescent lesions were resected during the surgeries; 62 of these lesions were confirmed as malignant on histopathology (i.e., true positives). Strikingly, 18 (29%) of these true positive lesions were not detected using standard inspection and/or palpation methods. Mean TBR was 4.4 (SD = 1.46; range: 1.7–9.8), and TBR generally decreased with increasing doses, likely due to increased background signal. TBR was constant throughout the surgical procedure, and fluorescence could be detected for at least 6 hours after infusion. Importantly, using NIR fluorescence enabled us to detect malignant lesions up to 8 mm below the tissue surface.

**Figure 3.** Intraoperative detection of ovarian cancer metastases using fluorescence-based imaging. A and B, color (left column), fluorescence (middle column), and merged (right column) images of retroperitoneal lymph nodes containing metastases of ovarian cancer (A) and superficial peritoneal metastases of ovarian cancer (B).
showing the added value of using light in the NIR spectrum (Supplementary Movie S1).

Histopathology of resected lesions
No malignant disease was found in 21 of the 83 fluorescent lesions, corresponding to a false positive rate of 23%. These false positive lesions were observed primarily in lymph nodes, representing 52% of all false positive lesions (Supplementary Table S5). Additional staining experiments and a closer examination of these lymph nodes revealed that activated macrophages, accumulated in the sinuses of the lymph node, express folate receptor beta (FRβ), which is also a binding target for OTL38. Our immunofluorescence experiments revealed that the fluorescence signal colocalized with FRβ staining (Fig. 4). Other false positive results arose due to the expression of FRα on the apical membrane of noncancerous epithelial cells in the uterus and fallopian tubes, which are routinely resected during cytoreductive surgery. The mean TBR of the false positive lesions was 5.4 (SD = 2.0, range: 1.8–9.3), which did not differ sufficiently from true positive lesions (mean = 4.4, range: 1.7–9.8) to allow us to differentiate between false positive and true positive lesions based solely on TBR. We observed only two false negative lesions. Finally, fluorescent microscopy revealed the accumulation of OTL38 in the membrane and cytoplasm of FRα-expressing tumor cells (for representative images, see Fig. 5).

Visual detection
The examination of tumor deposits based on color images obtained from the intraoperative videos allowed us to identify an average of 8.3 (SD = 5.4, range: 1–18) lesions per image. In contrast, performing the same assessment using the matching NIR fluorescence images allowed us to identify an average of 17.6 (SD = 10.8, range: 5–45) lesions per image, reflecting a more than 2-fold improvement in our ability to detect tumor lesions (Fig. 2).

Practical evaluation
The use of fluorescence imaging did not interfere with the surgeon’s ability to perform cytoreductive surgery, and the majority of participating surgeons reported that they found the technique to be useful (Supplementary Materials and Methods).

Discussion
Here, we report the successful use of the first tumor-specific NIR fluorescence–based imaging agent to target FRα in ovarian cancer, significantly increasing removal of tumor lesions.

In healthy volunteers, OTL38 caused moderate hypersensitivity; however, these reactions were easily managed. Given their symptomology, these reactions were likely pseudoallergic, a finding that has been described previously by Szebeni with respect to radiocontrast media (25). Moreover, investigating the cause of this hypersensitivity led to the development of procedures designed to minimize or eliminate this reaction in our subsequent study with cancer patients. This was likely related to aggregation of OTL38 rather than a classic allergic response to the drug, suggesting that the severity may be reduced further by modifying the drug’s formulation. Regardless, even these reactions were not severe enough to preclude the administration of a single dose of OTL38.

In healthy volunteers, the agent was essentially cleared from the plasma within 2 to 3 hours of intravenous delivery; however, a stable signal remained visible in the skin for at least 6 hours after dosing. This information was extremely valuable for determining the optimal time window for intraoperative imaging in patients, in which a favorable TBR was required during the surgical procedure. Our assessment of TBR at all doses revealed that the TBR of fluorescent lesions was maintained throughout the surgical procedure (i.e., 2 to 6 hours after dosing). However, because the fluorescent lesions were resected during surgery, we were unable to track the TBR of individual lesions over time.
A sufficiently high TBR is needed to optimally detect the tumor; in our study, a TBR of approximately 4.4 allowed the clear detection of tumor deposits. Higher doses (nonquenched) of OTL38 may translate into higher TBR, assuming linear binding of the agent to the tumor and background tissue. However, because the tumor contains a fixed number of receptors, it is conceivable that even with a low dose, the majority of FRα molecules in the tumor tissue will be bound by the agent. Therefore, higher doses will not necessarily increase the tumor-specific signal but might lead to increased nonsaturable, nonspecific background binding, resulting in a less favorable TBR at higher doses. Indeed, the highest dose used in this study (0.05 mg/kg) resulted in a high background signal and lower TBR value, whereas the lowest dose tested (0.0125 mg/kg) yielded the highest TBR and, most importantly, the mildest symptoms. To obtain the best imaging results, the exposure times differed between the different dosing groups; thus, the lowest dose (0.0125 mg/kg) required the longest exposure time (75 ms). Nevertheless, even this relatively longer exposure time enabled us to perform real-time imaging.

When translated to our patient cohort, the optimal dose of OTL38 (i.e., 0.0125 mg/kg) enabled the surgeons to successfully identify malignant lesions with reasonably high sensitivity and specificity. Moreover, 29% of all resected malignant lesions would have gone undetected without the aid of fluorescence-based imaging. Unfortunately, the relatively low number of patients precluded our ability to calculate the specificity and sensitivity of the technique. Moreover, our inability to study true negatives precluded a clear assessment of specificity. Nevertheless, both the in situ and ex vivo visual detection of lesions were clearly improved by the use of fluorescence-based imaging. With respect to in situ detection, 29% more lesions were resected. However, even this increase may underestimate the total number of lesions that could be detected during surgery, as resection is dependent upon several factors other than detection. Finally, our ex vivo visual detection was performed using still images, as it was not feasible to count lesions during surgery. Although this approach is commonly used and yields useful information, it may be considered suboptimal, as three-dimensional and tactile information is lost.

Although epithelial ovarian cancer cells overexpress FRα, this receptor is also expressed, albeit to a lesser extent, at the apical membrane of various noncancerous epithelial cells. During surgery, we noted mild, homogenous fluorescence of the uterus and the fallopian tubes, and biopsy revealed FRα expression in these nonmalignant tissues, consistent with previous reports (17, 26). The fluorescence signal in these tissues was homogenous and was clearly distinguishable from the fluorescence measured in the tumor deposits.

In the majority of our patients, we detected brightly fluorescent lymph nodes, and only a small number of these lymph nodes actually contained ovarian cancer metastases. The fluorescence measured in the noncancerous lymph nodes was likely due to OTL38 binding to activated macrophages, which express FRβ (27–30). However, the formation of OTL38 aggregates and
nonspecific uptake by lymph nodes is unlikely, as dissolving the agent at 7.5 μmol/L in 5% dextrose did not lead to the formation of measurable aggregates. Indeed, given that only 3 of the 12 patients received the highest dose (0.05 mg/kg), corresponding to a molarity slightly higher than 7.5 μmol/L (9.5 to 11.8 μmol/L OTL38), the presence of aggregates in the infusion solution is highly unlikely. In addition, to further minimize the likelihood of aggregate formation in the circulation, the solution was infused for 60 minutes. Although this apparent false positive fluorescence in the lymph nodes could be considered a drawback of this imaging agent, activated macrophages may actually be tumor-associated macrophages that play a role in preparing the tumor environment for metastasis (31–33). This notion is supported by our finding that FRβ-expressing macrophages were also found in primary tumors and in lymph nodes that did contain metastasized tumor cells (Supplementary Fig. S7). Until the precise role of FRβ-expressing macrophages in the lymph nodes is determined, lymph nodes should be resected solely on the basis of standard clinical assessments.

The use of fluorescent light in the NIR spectrum allowed us to detect lesions beneath the tissue surface, which is a major improvement over agents that use light outside the NIR spectrum (23, 34, 35). For example, in our study, malignant lesions were visible up to approximately 1 cm below the tissue surface. In addition, most biologic tissues have extremely low autofluorescence when excited by light in the NIR spectrum (36, 37). OTL38 also has several advantageous pharmacokinetic properties, including long residence time in the tumor and relatively rapid clearance from plasma; these properties provide the surgeon with a long window of time in which the tumor lesions can be detected. Unlike fluorescent antibodies, which have a much longer terminal half-life, OTL38 can be administered shortly before surgery (38). Although more tumor deposits can be visualized and resected using intraoperative fluorescence imaging with OTL38 compared with conventional methods, more prospective research is necessary to establish the effect on overall survival. In addition, the diagnostic accuracy of fluorescence imaging with OTL38 should be further assessed in a larger patient group.

In conclusion, we provide the first evidence that a specific intraoperative NIR imaging agent can be used to increase the efficacy of tumor removal in patients with ovarian cancer. Our approach to clinical translation using both healthy subjects and patients in the same phase I protocol allowed us to rapidly determine the optimal dose, formulation, and time window for intraoperative imaging, thereby greatly increasing the level of cytoreduction achieved in patients with ovarian cancer.

Disclosure of Potential Conflicts of Interest

A.F. Cohen is a consultant/advisory board member for Omnicomm. P.S. Low is an employee of and has ownership interest (including patents) in On Target Laboratories. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.E.S. Hoogstins, Q.R.J.G. Tummers, K.N. Gaarenstroom, C.D. de Kroon, T. Bosse, J. Vuyk, J. Burggraaf

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.E.S. Hoogstins, Q.R.J.G. Tummers, J.B.M.Z. Tribbos, A.F. Cohen, J. Burggraaf, A.L. Vahrmeijer

Writing, review, and/or revision of the manuscript: C.E.S. Hoogstins, Q.R.J.G. Tummers, K.N. Gaarenstroom, C.D. de Kroon, J.B.M.Z. Tribbos, T. Bosse, V.T.H.B.M. Smit, J. Vuyk, C.J.H. van de Velde, A.F. Cohen, J. Burggraaf, A.L. Vahrmeijer

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.E.S. Hoogstins, Q.R.J.G. Tummers, J. Burggraaf


Acknowledgments

The authors thank all of the healthy volunteers and patients who participated in the study. The authors also thank Dr. Mark Boomstra, Dr. Noor Rooger, and Dr. Hein Handgraaf for their assistance; Dr. Jogchum Belman for assistance during the surgical procedures; Margriet Löwik and Dorien Berends-van der Meer for assistance during the patient inclusion process; and Marietje Prevo and Brendy van den Akker for assistance with the IHC and fluorescence microscopy.

Grant Support

The Centre for Human Drug Research (a not-for-profit foundation) and the Leiden University Medical Center received financial compensation, the study drug, and equipment for performing this study from On Target Laboratories LLC.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 30, 2015; revised February 23, 2016; accepted March 14, 2016; published online June 15, 2016.

www.aacjrournals.org

Clin Cancer Res; 22(12) June 15, 2016 2937

References

A Novel Tumor-Specific Agent for Intraoperative Near-Infrared Fluorescence Imaging: A Translational Study in Healthy Volunteers and Patients with Ovarian Cancer

Charlotte E.S. Hoogstins, Quirijn R.J.G. Tummers, Katja N. Gaarenstroom, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/22/12/2929

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2016/07/14/22.12.2929.DC1

Cited articles
This article cites 38 articles, 8 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/22/12/2929.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.