Safety, anti-tumor activity and T-cell responses in a dose-ranging phase 1 study of the oncolytic peptide LTX-315 in patients with solid tumors

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Trial registration
ClinicalTrials.gov NCT01986426
Statement of translational relevance

A vital mechanistic step underlying the activity of immune-mediated cancer therapy is effective exposure of danger-associated molecular pattern molecules (DAMPs) and tumor antigens. LTX-315 is a membranolytic peptide that induces immunogenic cell death. It has demonstrated a marked impact on the tumor microenvironment in preclinical studies. In this phase I trial, we show that intratumoral injection of LTX-315 is well tolerated, has clinical activity including the induction of abscopal responses, and leads to intra-tumoral effects including an increase of infiltrating CD8+ T cells.
ABSTRACT

Purpose
LTX-315 is a first in class, 9-mer membranolytic peptide that has shown potent immunomodulatory properties in preclinical models. We conducted a phase 1 dose-escalating study of intratumoral LTX-315 administration in patients with advanced solid tumors.

Patients and methods
Thirty-nine patients were enrolled, receiving LTX-315 injections into accessible tumors. The primary objective was to assess the safety and tolerability of this approach, with anti-tumor and immunomodulatory activity as secondary objectives. Tumor biopsies were collected at baseline and post-treatment for analysis of immunological parameters.

Results
The most common treatment-related grade 1-2 adverse events were vascular disorders including transient hypotension (18 patients, 46%), flushing (11 patients, 28%), and injection site reactions in 38% of patients. The most common grade 3 LTX-315-related toxicities were hypersensitivity or anaphylaxis (4 patients, 10%). Analysis of immune endpoints in serial biopsies indicated that LTX-315 induces necrosis and CD8+ T cell infiltration into the tumor microenvironment. Sequencing of the TCR repertoire in peripheral blood identified significant expansion of T-cell clones after treatment, of which 49% were present in available tumor biopsies after treatment, suggesting that they were tumor-associated. Substantial volume reduction (≥30%) of injected tumors occurred in 29% of the patients, and 86% (12 out of 14 biopsies) had an increase in intralesional CD8+ T cells post-treatment. No partial responses by immune-related response criteria were seen, but evidence of abscopal effect was demonstrated following treatment with LTX-315.

Conclusion
LTX-315 has an acceptable safety profile, is clinically active, induces changes in the tumor microenvironment and contributes to immune-mediated anticancer activity.
INTRODUCTION

Local intratumoral immunotherapy of cancer aims to induce local and systemic immune responses via direct injection of immunostimulatory agents. This should induce tumor cell lysis, followed by the release of tumor-derived antigens and subsequent activation of tumor-specific effector T cells. Other advantages of direct intratumoral therapy include enhanced efficacy and bioavailability of the agent at the local injection site, limited systemic exposure and off-target toxicities, as well as efficient use of drug dosage (1-3).

The oncolytic peptide LTX-315 is a 9-mer peptide designed by structure-activity relationship studies from the host defense peptide lactoferricin (4,5). It has demonstrated the ability to kill human cancer cells of diverse origin (6). LTX-315 exerts its activity through a membranolytic effect and is equally active against both drug-resistant and drug-sensitive cancer cells. Selectivity for cancer cells results from the cationic amino acid side chains of LTX-315 interacting with the abundant anionic membrane components present at higher levels on cancer cells than in normal cells (7-9).

The oncolytic effect of LTX-315 involves perturbation of both plasma and mitochondrial membrane integrity with subsequent release of danger-associated molecular pattern molecules (DAMPs) and tumor antigens. When injected locally into tumors established in immunocompetent mice, LTX-315 induces complete regression and specific anticancer immune responses with subsequent long-term protective immunity (6,10,11). Treatment with LTX-315 in these models results in an increase of CD8\(^+\) tumor infiltrating T cells (TILs), and reprogramming of the tumor microenvironment including decreases in the local immunosuppressive regulatory T-cell and myeloid-derived suppressor cell populations (6,10,12,13). In an experimental rat sarcoma model, LTX-315 induced an abscopal effect as demonstrated by regression of distal non-treated lesions, associated with significant infiltration of CD8\(^+\) T-cells in both treated and non-injected lesions (11).

We have conducted a phase 1 dose-escalating study to assess the safety, tolerability, maximum tolerated dose, pharmacodynamics and anti-tumor activity of single agent intratumorally administered LTX-315 in patients with advanced solid tumors and at least one transdermally accessible lesion.
MATERIALS AND METHODS

Study design

The study was an open-label multicenter study of LTX-315 with multiple intralesional injections in patients with solid tumors for whom alternative treatment options were not available or suitable (NCT01986426). The primary objective of the study was to assess the safety and tolerability of intratumoral administration of different LTX-315 doses and concentrations. Patients with any solid tumor were eligible for recruitment into the study. As secondary objectives, the anti-tumor activity by immune-related response (irRC) (14), and pharmacokinetic (PK) profile of LTX-315 were evaluated. Blood samples and biopsies of injected tumor were analysed for pharmacodynamic endpoints.

LTX-315

Manufacturing of LTX-315 was carried out at Bachem (Bubendorf, Switzerland) in accordance with Good Manufacturing Practice (GMP). LTX-315 is a powder for injection formulated by reconstitution in 0.9% saline solution and is administered by intratumoral injection either under direct vision or with ultrasound guidance. In the first in man study with LTX-315 (15), 2 patients experienced hypotension following first injection with LTX-315 doses ≥8 mg. This limited the dose of LTX-315 per injection to 7 mg in the current study. The total dose of LTX-315 to be administered was determined by dose escalation cohort. Two to 7 mg of LTX-315 was administered per injection at concentrations of 10 or 20 mg/ml. Three treatment schedules were tested sequentially (Figure 1A-C and Supplementary Table 1A). To break local immune tolerance and build an effective and sustainable systemic immune response, frequent and multiple doses of LTX-315 were administrated during the first weeks of therapy followed by maintenance with less frequent administrations. In the initial schedule (Arm A), LTX-315 was administered to a single lesion on days 1, 2 and 3 during the first week, and thereafter once weekly for a total of 6 weeks. The maintenance phase included one injection per day every two weeks for 20 weeks, or until disease progression. A second and third lesion could be treated, and these were injected sequentially, exactly as for lesion 1, in weeks 7 to 12, and then weeks 13 to 18, respectively. Only the last injected lesion was treated in the maintenance phase. This treatment schedule (Arm A) included one injection twice-daily (3 hour interval) on each dosing day in the three first cohorts (2, 3 and 4 mg per injection, respectively). Since no Grade 2-4 adverse events were observed after two injections per day (2+2 mg = 4 mg, 3+3 mg = 6 mg and 4+4 mg = 8 mg), the following cohorts 4-7 included one injection at each dosing day instead of two with 4 mg day 1 and either 5, 6 or 7 mg per injection the following days (Supplementary table 1A). To allow treatment of multiple lesions per patient, the schedule in Arm B involved either one injection into multiple lesions in parallel on the same dosing day, at days 1, 2 and 3 during the first week, followed by one injection to each lesion in week 2, week 4 and then every 3 weeks until week 49 or progressive disease (Arm B*), or injection of multiple lesions on the same dosing day, at days 1, 2, 8, 9, 15 and 16 (Arm B**) (Figure 1A-C). In the latter schedule, multiple injections per lesion were included per dosing day to compensate for the reduced number of dosing days, and these...
were separated by 5-minute intervals and injected radially in a clockwise manner. The number of injections per lesion was amended to be dependent upon the lesion size. Arm B** evolved from Arm A and B* to address emerging hypersensitivity concerns with extended exposure to LTX-315. A maximum of 12 injections were given per dosing day.

**Patients**

Patients evaluable for anti-tumor response received at least one injection of LTX-315 and had at least one post-dosing tumor evaluation. Eligible patients were aged over 18 years, had unresectable, advanced or metastatic disease (any solid tumor type), and had received all appropriate available standard of care therapies, or were not eligible or suitable for such treatment. All patients had at least one lesion accessible for injection (cutaneous, subcutaneous, oral or lymph node) which was between 1 and 9 cm in longest diameter, and another non-injected target lesion that was measurable by CT. All patients were required to have ECOG (Eastern Cooperative Oncology Group) performance status of 0 or 1, normal cardiac function, and baseline blood laboratory parameters indicating adequate bone marrow, kidney and liver function. Patients with a history of systemic autoimmune disease requiring anti-inflammatory therapy or immunosuppressive therapy within the last 3 months prior to study drug administration, incomplete recovery from adverse events (AEs) due to prior therapies, and those with active or unstable cerebral metastases were excluded from the study. Ethical approval for this Phase 1 study was obtained from the Regional Committee of Medical Research Ethics, South-Eastern-Norway (no 2013/1650), and from the UK NHS Health Research Authority (no 13/SC/0467). Written informed consent was obtained from patients prior to treatment and the study was conducted in compliance with Good Clinical Practice (GCP) guidelines after review and approval by appropriate Research Ethics Committees.

**Tumor biopsies**

One to 3 core needle biopsies of the tumor were taken before the first treatment and at week 4 or 7 (contingent on protocol version and Arm). The biopsies were embedded in paraffin and stained with hematoxylin and eosin (H&E) for quality assessment and evaluation of necrosis, followed by immunohistochemical (IHC) staining for tumor microenvironment parameters.

**Assessments and endpoints**

**Safety outcomes**

Treatment-emergent adverse events (TEAEs) were defined as all AEs occurring on or after the first dose of study medication up to 30 days after the last dose. Treatment-related (TR) AEs, deemed by the investigator to be related to LTX-315 were tabulated. Adverse events were summarized by frequency and percentage of patients and categorized by MedDRA version 16.1 system organ class and preferred term unless otherwise indicated and graded according to NCI-CTCAE (version 4.0). Serious adverse events (SAEs), TEAEs leading to treatment discontinuation and death were also assessed.
Anti-tumor activity

Overall response was calculated based on the scheduled full tumor assessments by computed tomography/magnetic resonance imaging (CT/MRI) of non-injected lesions. Tumor responses were assessed based on irRC (14). The baseline scan was performed within 28 days prior to the first treatment. In addition to the overall tumor response, change in size was also calculated for individual lesions. A new clinical response criterion has previously been designed to address the unique needs of intratumoral immunotherapy. Intratumoral Response Evaluation Criteria in Solid Tumors (iTRECIST) provides guidelines for assessing responses in injected and non-injected lesions as treatment evolves (16). However, these guidelines were not available during the course of the study described herein and therefore not used as an analytical tool in this study.

Pharmacodynamic analyses

Immunohistochemistry. All tumor biopsies were subjected to IHC to assess CD8 expression. Staining was performed on slides using the Roche Ventana Benchmark XT autostainer. After antigen retrieval, staining was performed using CD8 antibodies (M7103, clone C8/144B, or HD-FG-000019) and detected with secondary antibody using Ultraview Universal DAB detection kit. Image acquisition was performed using the Hamamatsu Nanozoomer XR scanner. HalioDx Digital Pathology Platform was used for quantification of CD8 density in the tumor area.

Biopsies from a subset of patients were evaluated by Immunoscore® analysis that included staining with anti-CD3 (HD-FG-000013) and anti-CD8 (HD-FG-000019) antibodies on consecutive slides along with digital quantification of T-cell infiltrate (HalioDx, Marseille, France).

TCR repertoire analysis. T-cell receptor (TCR) beta chain CDR3 regions were sequenced by ImmunoSeq™ (Adaptive Biotechnologies, Seattle, WA), with primers annealing to V and J segments, resulting in amplification of rearranged VDJ segments from each cell. Clonality values were obtained through the ImmunoSeq Analyzer software (www.adaptivebiotech.com/immunoseq/analyzer). Differential abundance analysis was assessed to identify clones that were significantly expanded or contracted from baseline to post-treatment time points. Significantly expanded clones were identified in peripheral blood and then compared to those in post-treatment tumor samples to determine expansion of tumor-associated T-cell clones.

Gene expression analysis. Identification of upregulated key immune genes involved in tumor regression post-treatment was carried out by hierarchical clustering of Immunosign21® gene signature (HalioDx, Marseille, France) by means of a predefined set of genes, including those relating to effector T cells, T-helper type 1 (Th1) cells, chemokine, and cytokine function (17).

Pharmacokinetics

Plasma was collected before LTX-315 injection and at 1 minute, 5 minutes, 30 minutes, 60 minutes and 2 hours post-dose. Actual sampling time was used to calculate pharmacokinetic parameters. The
concentrations of LTX-315 in human plasma samples were measured using LC-MS/MS (API5500 Mass Spectrometer). The samples were analyzed over the calibration range of 2.00-500 ng/mL. Pharmacokinetic data parameters were derived using Phoenix™ WinNonlin® 8.1 (Certara, USA, Inc.).

Statistics
This was a phase I study with variable numbers of patients per cohort. No statistical justification has been applied to the number of patients required. In general, statistical analyses were descriptive. Data summaries included frequency counts and percentages for categorical measures and n, mean, standard deviation, median, minimum and maximum values for continuous measurements. All confidence intervals (CIs) were 2-sided. Baseline assessment is the last assessment within 28 days before the first dose of study treatment. Baseline could be the same day as first treatment administration.

RESULTS
Patients
Thirty-nine patients with solid tumors were treated with LTX-315 (Table 1) and evaluated for safety. The median age was 60 years (range 30-80 years). Fifteen patients (38%) were male and 24 patients (62%) were female. Median duration of disease since diagnosis was 60 months (range 13 to 234 months).

Safety
Thirty-nine patients received at least one dose of LTX-315 and were eligible for analysis of the primary safety endpoint. Aggregated treatment-related AEs reported during the study are presented in Table 2, and toxicity data according to arm and cohort is shown in Supplementary Table 1B-C. The most common LTX-315-related toxicities were vascular disorders including grade 1-2 hypotension (18 patients, 46%), flushing (11 patients, 28%), and injection site reactions including pruritus (6 patients, 15%), rash (6 patients, 15%), paresthesia (4 patients, 10%) and local pain (2 patients, 5%). Hypotension occurred as an acute effect immediately after intratumoral injection, and in almost all cases was an isolated finding unassociated with features of hypersensitivity. SAEs related to LTX-315 were uncommon and occurred in 4 patients (10%). One patient experienced a grade 4 toxicity of hypersensitivity related to LTX-315 treatment. Clinically significant hypersensitivity reactions were seen following ≥10 weeks of multiple 5 to 7 mg doses of LTX-315 (in 3 out of 14 patients on treatment for this length of time), and when this did occur it was an acute event immediately following an intratumoral injection. Treatment was discontinued in each case following a hypersensitivity reaction. No deaths related to treatment occurred. The treatment schedule was amended to include implementation of prophylactic therapy for hypersensitivity with the use of antihistamines and a leukotriene receptor antagonist, together with a shorter treatment duration of 3 weeks. Following the introduction of these mitigating steps there were no further discontinuations due to hypersensitivity events. Nine out of 39 patients (23%) experienced grade ≥ 3 events related to LTX-315. Some patients experienced grade ≥ 3 events in more than one category. All toxicities were manageable and reversible.
Anti-tumor activity

We assessed anti-tumor activity using irRC in 27 evaluable patients in all treatment arms, by considering the total tumor burden excluding the injected lesion (Figure 1D). A patient with melanoma had a 48% reduction in tumor burden from baseline, close to the threshold for partial response criteria of 50% regression. Another patient with a progressing retromammary desmoid tumor experienced progressive symptom relief and long-term disease stabilization (>2.5 years) after 16 weeks of treatment, indicating clinical benefit. Prior treatment with IFN-α resulted in slowing of tumor growth but treatment was discontinued due to pneumonitis. We believe it is reasonable to attribute the subsequent sustained stable disease to LTX-315 (18). The patient did not have a non-injected lesion evaluable for response and is therefore not included in this analysis. Forty-four percent (12/27) of the patients achieved irSD, while 56% (15/27) experienced irPD as best response (Figure 2A). Although no objective responses by irRC were seen in patients treated with LTX-315, shrinkage of distant non-injected lesions (abscopal effect) by up to 82% in a single lesion was observed (Figure 2A and supplementary Table 2). A patient treated in a gluteal leiomyosarcoma lesion had 66% regression of a non-injected lung lesion (Figure 2B). Seven out of 24 evaluable patients (29%) had >30% regression in at least one injected lesion.

Pharmacokinetics

Samples from 34 out of 39 patients (87%) were available for PK analysis. Transient systemic exposure to LTX-315 was detectable in all but 2 of 34 patients despite the intratumoral route of injection, and was highly variable. Across the cohorts, T_max ranged from 0.02 to 0.79 h, and C_max ranged from 6 to 890
ng/ml (Table 3). In most patients, plasma concentration declined rapidly to low or undetectable levels within 1h. Inadequate numbers of serial samples were available for an analysis of half-life.

**Pharmacodynamics**

*Analysis of tumor microenvironment including T-cell infiltration in injected tumors*

Paired pre- and post-treatment tumor biopsies were available in 14 out of 27 (52%) patients. Only patients with a CT scan after treatment were included for correlation of immune response to clinical benefit. The abundance of intratumoral CD3+ TILs increased after LTX-315 in all patients evaluated, irrespective of the level of TILs in the tumor microenvironment before treatment (Figure 3A). Twelve out of 14 (86%) of those paired biopsies showed an increase in CD8+ TILs. Quantitative assessment of biopsies from 5 representative patients is shown (Figure 3B).

*Upregulation of key genes involved in immune tumor rejection*

To further investigate the effect of LTX-315 on the tumor microenvironment, and on TILs in particular, gene expression analysis was performed in paired tumor material. Available pre- and post-treatment biopsies with most marked increase in TILs were included in an array analysis. The expression of a panel representing 21 pivotal genes (Immunosign® CR 21) necessary for mounting an efficient adaptive anti-tumor immune response was assessed. This panel includes genes involved in activation of cellular immunity, as well as markers of key immune cell subsets (especially cytotoxic T cells and Th1 cells), signaling molecules and receptors. Expression signatures showed that the majority of these genes were upregulated post-treatment, indicating that LTX-315 enriches the tumor microenvironment with adaptive immune components (Figure 3C).

*Expansion of tumor-associated T-cell clones*

Changes in T-cell clonality in blood were studied in a subset of patients. The frequency of each T-cell clone was examined in pre- and post-treatment blood samples. To identify those T-cell clones whose frequencies differed between pre- and post-treated samples, an algorithm described by DeWitt et al. was used (19). T-cell clones with significant clonal expansion were identified and counted (Figure 3D and supplementary Figure 1A). In healthy individuals, an average of 16 T-cell clones have been reported as significantly expanding in a 4-week interval (20). The number of significantly expanded T-cell clones in the patients was above the mean number found in healthy individuals in 4 out of 6 (67%), indicating that LTX-315 induces a systemic immune response. A median of 49% of the T-cell clones significantly expanding in blood were also detected in the patients’ corresponding tumor specimen post-treatment (Supplementary Figure 1B). By contrast, the median proportion of significantly expanded T-cell clones in blood also represented in the tumor before treatment was only 23%. The median number of T-cell clones that significantly contracted in blood and present in tumor pre- and post-treatment were 17% and 3%, respectively. Taken together, these data suggest that many of the T-cells clones expanding in patient blood post-treatment are likely to be associated with components of the tumor.
DISCUSSION

Intratumoral immunotherapy is emerging as an effective treatment strategy within the immuno-oncology field. Preclinical data supports the hypothesis that by addressing the tumor microenvironment and heterogeneity directly in this way, it may be possible to induce efficient local and systemic tumor-specific immune responses (2,21-24). Talimogene laherparepvec (“T-VEC”) was an early intratumoral therapy to receive marketing approval, and many other locally administered therapies with diverse mechanisms of inducing systemic anti-cancer immunity have entered clinical development (25). In this trial we show that intratumoral immunotherapy with LTX-315 is well tolerated and results in the induction of local and systemic tumor-specific immune responses. The primary objective of this trial was to explore the safety and tolerability of LTX-315. Thirty-nine patients were treated with intratumoral injections of LTX-315. The safety data demonstrate that transient grade 1-2 hypotenion and administration site reactions were common but readily reversible. Hypotension was likely a result of LTX-315 binding to the MRGx2 receptor on basophils in the intravascular space. Binding of cationic antimicrobial peptides to the MRGx2 receptor induces release of histamine, which can result in vasodilator effects (26). Systemic hypersensitivity reactions after extended exposure to LTX-315 for 10 weeks or more was the most clinically significant toxicity (Table 2). This was effectively mitigated by introducing prophylactic pre-medication with antihistamine and leukotriene receptor antagonists, which was effective in preventing recurrence after rechallenge, and by limiting the duration of exposure to multiple injections. It appears that prolonged dosing is the key driver for any hypersensitivity-/anaphylaxis-related AE since clinically significant hypersensitivity to LTX-315 was experienced after >10 weeks of treatment. Moreover, a changed LTX-315 posology including a 3-week dosing schedule and maximum 5 mg per injection was well tolerated.

A number of patients experienced significant tumor regression in injected lesions. Substantial volume reduction (≥30%) of injected tumors occurred in 29% of the patients. More importantly, abscopal responses in distant untreated tumor deposits occurred in several patients (Figure 2A-B and Supplementary Table 1), consistent with the concept of local tumor lysis giving rise to a systemic anticancer immune response (2,22). By inducing immunogenic cell death locally in all cancer cells independent of resistance phenotype, LTX-315 can expose a repertoire of tumor antigens and endogenous danger signals to the immune system, generating a systemic tumor-specific immune response.

An important secondary objective of this trial was to study the immunomodulatory activity of LTX-315. In serial tumor biopsies before and after LTX-315 treatment we demonstrated an influx of CD3+ and CD8+ TILs to the injected lesions independent of tumor microenvironment status before treatment (Figure 3A-B). An increase in CD8+ T cells was observed in 12 out of 14 evaluable patients (86%). The presence of TILs, and especially CD8+ T cells, has been recognized as a predictive marker for anti-tumor immune responses in several indications (27-31). LTX-315 induces release of immunostimulants such
as ATP, calreticulin, HMGB1 and cytochrome c from cancer cells (32-34). These damage-associated molecular patterns (DAMPs) activate immature dendritic cells and are essential in mounting potent systemic anticancer immune responses, including the infiltration of TILs into the tumor microenvironment (35). As reported previously, this demonstrates that LTX-315 can create local immune responses that correlates with clinical benefit in patients (18), presumably resulting from treatment-induced release of DAMPs and tumor antigens.

Gene expression analysis of tumor biopsies from a subset of patients in this trial has highlighted the ability of LTX-315 to enrich the tumor microenvironment with adaptive immune components in cancer patients. Substantial changes in injected lesions were demonstrated by a 21 gene mRNA expression panel, demonstrating activation of tumor-specific immune responses. This panel includes genes involved in a Th1 response and Th1 maturation, supporting CD8$^+$ T-cell functions. We observed a significant induction of gene expression involved in cytotoxic T-cell effector function such as perforin and granzymes (36,37). Transcriptomic analyses have confirmed a strong association between TIL profile and the expression of genes involved in Th1 response and maturation (38). Importantly, Th1 gene signatures have been correlated with good prognosis in several cancers including breast cancer (39) and melanoma (40).

Immunosequencing of TCR is a sensitive, highly accurate technology that quantifies and specifies the clonal distribution of lymphocytes in biological samples and enables new ways of analyzing treatment responses. Consistent with the LTX-315-mediated modulation of the tumor microenvironment (Figure 3C), we have demonstrated changes in peripheral T-cell clonality post-treatment. Here we show that 4 out of 6 patients demonstrate an expansion of circulating T-cell clones after treatment above the level reported in healthy individuals (26-126 significantly expanding T-cell clones) (Figure 3D and Supplementary Figure 1). A larger proportion of these expanding T-cell clones were present in post-treatment tumor tissue compared to pre-treatment tumor tissue, indicating that they were tumor-associated. Moreover, fewer of the significantly contracted T-cell clones in peripheral blood were present in tumor tissue post- and pre- treatment. Previous studies have shown that clonal T-cell expansion in response to immunotherapy correlates with clinical benefit (41,42).

Our clinical data also showed a correlation between TILs, gene expression analysis, the number of expanding T-cell clones, and the abscopal effect observed. In patient Melanoma #1, who had 82% regression in a non-injected lesion (Figure 2A), the biopsy from the injected lesion was completely necrotic when analyzed by IHC (Figure 3A). Furthermore, non-treated lesions from patient Sarcoma #3 and patient Breast cancer #4 showed regression of 66% and 54%, respectively (Figure 2A). Treated tumors from these two patients exhibited an increase in CD3$^+$ and CD8$^+$ TILs post treatment (Figure 3A), as well as a transition to a tumor microenvironment richer in adaptive immune components, as analyzed by Immunosign gene expression signature assessment (Figure 3C). Moreover, a significant
increase in the number and diversity of expanding T-cell clones in blood was also observed post treatment (Figure 3D and Supplementary Figure 1).

Recent developments in immunotherapy approaches have had significant clinical impact in the field of cancer treatment. Antibodies targeting the inhibitory receptors CTLA4 and PD1 have shown promising efficacy in several types of cancer (43). However, only subsets of patients experience clinical benefit and pre-existing TILs may contribute to clinical response (44). Patients lacking TILs may therefore require therapeutic interventions to prime the T-cell response to enable clinical benefit of checkpoint inhibitor therapy (45). The ability of LTX-315 to increase tumor T-cell infiltration and T-cell clonality therefore positions LTX-315 as a combination partner for other immunotherapies, including immune checkpoint inhibitors. In preclinical tumor models, combination of LTX-315 and an immune checkpoint inhibitor (anti-CTLA-4) demonstrated significant synergy (13). In this phase I trial we show that intratumoral injection of LTX-315 is well tolerated. The dosing regimen of LTX-315 should be further optimized to position LTX-315 as a therapeutic in combination with checkpoint inhibitors to address an unmet need in a selected group of indications. LTX-315-mediated upregulation of cellular immune components in the tumor microenvironment present a clear rationale for priming a response to checkpoint inhibitors (13). Interestingly, LTX-315 is also being developed as a technology to promote tumor-specific CD8+ T-cell infiltration into tumors, allowing their subsequent harvest, expansion in culture and infusion as an adoptive T-cell transfer regimen for soft tissue sarcoma (NCT03725605).
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Table 1. Summary of patient characteristics and previous ICI treatment.

<table>
<thead>
<tr>
<th>Tumour (n=39)</th>
<th>N</th>
<th>Patients with prior ICI treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>10</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Breast</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>H&amp;N</td>
<td>7</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>GI</td>
<td>3</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>Desmoid</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Primary vaginal cancer</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Carcinoma of unknown primary</td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>Age (median, range)</th>
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<tbody>
<tr>
<td>Male</td>
<td>38%</td>
</tr>
<tr>
<td>Female</td>
<td>62%</td>
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Median time since diagnosis (months) 60.3
Table 2. Summary of drug-related treatment-emergent adverse events occurring in at least 10% of patients or grade ≥ 3 in each arm.

<table>
<thead>
<tr>
<th></th>
<th>Arm A (N = 23)</th>
<th>Arm B (N = 16)</th>
<th>Arm A + Arm B (N = 39)</th>
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<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>Grade ≥ 3</td>
<td>Overall</td>
</tr>
<tr>
<td>Hypotension</td>
<td>15 (65.2%)</td>
<td>3 (18.8%)</td>
<td>18 (46.1%)</td>
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<td>Flushing</td>
<td>8 (34.8%)</td>
<td>3 (18.8%)</td>
<td>11 (28.2%)</td>
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<td>Pruritus</td>
<td>4 (17.4%)</td>
<td>2 (12.5%)</td>
<td>6 (15.4%)</td>
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<tr>
<td>Rash</td>
<td>3 (13.0%)</td>
<td>3 (18.8%)</td>
<td>6 (15.4%)</td>
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<tr>
<td>Musculoskeletal and connective tissue disorders</td>
<td>4 (17.4%)</td>
<td>3 (18.8%)</td>
<td>4 (10.2%)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>4 (17.4%)</td>
<td></td>
<td>4 (10.2%)</td>
</tr>
<tr>
<td>Hypersensitivity</td>
<td>4 (17.4%)</td>
<td>1 (4.3%)*</td>
<td>4 (10.2%)</td>
</tr>
<tr>
<td>Injection site paraesthesia</td>
<td>4 (17.4%)</td>
<td></td>
<td>4 (10.2%)</td>
</tr>
<tr>
<td>Paraesthesia</td>
<td>4 (17.4%)</td>
<td></td>
<td>4 (10.2%)</td>
</tr>
<tr>
<td>Anaphylactic reaction</td>
<td>3 (13.0%)</td>
<td>3 (13.0%)*</td>
<td>3 (7.7%)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>3 (13.0%)</td>
<td></td>
<td>3 (7.7%)</td>
</tr>
<tr>
<td>Tachycardia</td>
<td></td>
<td></td>
<td>3 (7.7%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td>2 (12.5%)</td>
<td>2 (5.1%)</td>
</tr>
<tr>
<td>Rash maculo papular</td>
<td></td>
<td>2 (12.5%)</td>
<td>3 (7.7%)</td>
</tr>
<tr>
<td>Local pain</td>
<td>1 (4.3%)</td>
<td>2 (12.5%)</td>
<td>3 (7.7%)</td>
</tr>
<tr>
<td>Syncope</td>
<td>1 (4.3%)</td>
<td></td>
<td>1 (2.5%)</td>
</tr>
<tr>
<td>Sepsis</td>
<td>1 (4.3%)</td>
<td></td>
<td>1 (2.5%)</td>
</tr>
</tbody>
</table>
Table 3. Pharmacokinetic of LTX-315 in human plasma. A summary of mean pharmacokinetic parameters for LTX-315. The different arms including distinct treatment schedules and LTX-315 doses per injection are shown.

<table>
<thead>
<tr>
<th>Arm</th>
<th>LTX-315 dose (mg/injection)</th>
<th>N</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>AUC h*ng/ml</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>3</td>
<td>24±26</td>
<td>6</td>
<td>0,08</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>6±1</td>
<td>NR</td>
<td>0,03</td>
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<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>95±104</td>
<td>28</td>
<td>0,02</td>
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<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>481±720</td>
<td>67±61</td>
<td>0,02</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>427±432</td>
<td>49±40</td>
<td>0,03</td>
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<tr>
<td></td>
<td>7</td>
<td>4</td>
<td>389±402</td>
<td>617±33</td>
<td>0,26</td>
</tr>
<tr>
<td>B*</td>
<td>3</td>
<td>3</td>
<td>59±26</td>
<td>22±19</td>
<td>0,25</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>0,03</td>
</tr>
<tr>
<td>B**</td>
<td>3</td>
<td>4</td>
<td>214±260</td>
<td>155±142</td>
<td>0,68</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>21</td>
<td>7</td>
<td>0,32</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>889</td>
<td>212</td>
<td>0,79</td>
</tr>
</tbody>
</table>

The different arms including distinct treatment schedules and LTX-315 doses per injection are shown. C<sub>max</sub> is the maximum (or peak) serum concentration of LTX-315 achieved after the drug was administrated. AUC is the integral of the concentration-time curve (after a single dose or in steady state). T<sub>max</sub> is the time to reach C<sub>max</sub>.
FIGURE LEGENDS

Figure 1. Treatment schedule and anti-tumor activity of LTX-315. (A) LTX-315 was injected in a single lesion on 3 consecutive days and subsequently on 1 dosing day weekly (induction phase). If the patient had another lesion available for injection, a second lesion was injected at week 7 and followed the same treatment schedule as the first lesion. Up to 3 lesions were treated per patient with this schedule (Arm A). Treatment was terminated with a maintenance phase of 1 dosing day every second week for a total of 20 weeks or to progressive disease. The starting dose for cohort 1 was 2 mg twice per day (4 mg total daily dose) and was escalated to 3 and 4 mg in cohort 2 and 3, respectively. In the following cohorts in Arm A, LTX-315 was injected once daily, with 4 mg on first dosing day followed by 5, 6, or 7 mg in the subsequent cohorts. (B) The treatment schedule was amended during the trial to include injections in multiple lesions on the same dosing day (Arm B*) at day 1, 2 and 3 during the first week, followed by 1 injection to each lesion in week 2, 4 and then every 3 weeks until week 49 or progressive disease. (C) The second part of Arm B (Arm B**) included injection of multiple lesions on the same dosing day, at days 1, 2, 8, 9, 15 and 16. LTX-315 dose was 3, 4 or 5 mg per injection, and the volume (30% of the tumor volume) and number of injections to each lesion was dependent upon the lesion size. (D) Spider plot showing tumor growth or shrinkage from baseline. The black lines show patients that experienced stable disease assessed by irRC as best response. The best responding patients, melanoma patient #1 and breast cancer patient #4, are indicated.

Figure 2. LTX-315 induces abscopal effects

(A) Best response at any time point after LTX-315 treatment in a single non-injected lesion (black bar) and overall response according to irRC (gray bar) in all patients treated with LTX-315 monotherapy that had CT scans at baseline and after treatment. Each patient’s tumor type and best overall response of the total tumor burden by irRC are annotated (SD = stable disease; PD = progressive disease). * indicated injected lesion size not assessed. (B) Abscopal effect in lung metastatic disease of a patient with recurrent metastatic leiomyosarcoma (sarcoma #3). The patient was enrolled in Arm A and received LTX-315 in the gluteal muscle lesion. Seven weeks after initiation of treatment one lung lesion had decreased by 64% (product of the 2 longest perpendicular diameters) from baseline.

Figure 3. LTX-315 administration enriches the adaptive immune component of the tumor microenvironment, and induces a polyclonal tumor-associated T-cell response in the periphery.

(A) CD3 and CD8 expression in baseline and post-treatment biopsies from injected lesions in 3 patients enrolled in Arm A. Qualitative assessment of tumor tissue collected from Melanoma #1 showed necrosis after treatment, while Sarcoma #3 and Breast cancer #4 showed an increase in TILs after treatment. (B) Quantification of CD3+ TILs in injected lesions at baseline and 4 weeks after start of LTX-315 treatment. The number of CD3+ T cells in tumor tissue from patients enrolled in Arm B** is shown. (C) LTX-315 induces upregulation of key genes involved in immune mediated tumor regression, including markers...
of T-cell activation and cytotoxicity. Heatmap showing the expression level of immune response-related transcripts in paired tumor biopsies collected from injected lesions from selected Arm A patients, at baseline (B) and 7 weeks (W7) after start of LTX-315 treatment. Red indicates high expression, blue indicates low expression. (D) Sequencing of the TCR repertoire in blood before and 7 weeks after treatment for selected patients enrolled in Arm A reveals significant expansion of numerous T-cell clones (shown by the gray). The number of significantly expanding T-cell clones is indicated at top of the bar. The presence of the same T-cell clones in tumor after treatment is shown in the black bar indicating that many of these T-cell clones are tumor-associated. The dotted line shows the average number of significantly expanding T-cell clones found in healthy individuals in a 4-week interval.
REFERENCES


Figure 1

A) Week 1 2 3 4 5 6 7 13 15 17 19 20

Lesion 1

Induction phase

Maintenance phase

Arm A
Single lesion injection
Dose 2-7 mg/injection
1 injection per lesion bidaily or daily

Arm B*
Multiple lesions injected concurrently
Dose 3 or 4 mg/injection
1 injection per lesion daily

Arm B**
Multiple lesions injected concurrently
Dose, 3, 4 or 5 mg/injection
Number of injections dependent on lesion size

B) Week 1 2 4 7 10 13 ....... every 3 weeks until W 49

C) Week 1 2 3
Figure 2  A)
Figure 2 B)
Figure 3 B)

![Graph showing cell counts per mm² for different tumor types at baseline and W4.](image)
Figure 3 (C)
Clinical Cancer Research

Safety, anti-tumor activity and T-cell responses in a dose-ranging phase 1 trial of the oncolytic peptide LTX-315 in patients with solid tumors

James Spicer, Aurelien Marabelle, JEAN-FRANCOIS BAURAIN, et al.

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