REPORT
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HUMANISED NANOMEDICINES SELECTIVELY AIMED TO KILL TUMOR CELLS CXCR4+ FOR TREATMENT OF ACUTE MYELOID LEUKEMIA

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1. Project Summary

Current chemotherapy for acute myeloid leukemia (AML) is associated with severe toxicity and side effects due to lack of selectivity. Although most patients respond to chemotherapy, many of them experience relapses and ultimately succumb to the disease, with overall 5-year survival in adult patients being less than 30%. Therefore, new strategies are needed to enhance effectiveness and improve patient survival. The chemokine receptor CXCR4 is overexpressed in leukemic cells in more than a half of AML patients. This overexpression correlates with reduced overall and disease-free survival and increased resistance to chemotherapy. Prior to the start of this project, we had developed a novel protein nanoconjugate specifically targeted to CXCR4+ leukemic cells using the T22 ligand, conjugated with the potent antimitotic agent monomethyl auristatin E (MMAE). Our therapeutic strategy takes advantage of the CXCR4 overexpression in AML blasts compared to normal cells. The prototype of this nanoconjugate includes a GFP domain, which has been used for both in vitro and in vivo for location of the nanoparticle. Previously, we demonstrated that the T22-GFP-MMAE nanoconjugate exhibits significant antitumor effects in disseminated AML animal models generated with cell lines. The primary objective of this project has been to humanize the T22-GFP-MMAE nanoconjugate by replacing the GFP domain with a human protein to avoid immune reactions in patients. Additionally, we have utilized patient-derived xenografts (PDXs) to define the patient subpopulation that will benefit most from this therapy. Finally, we have employed humanized mouse models to study its effects on normal hematopoietic cells. As the final outcome of this project, we have developed a novel nanoconjugate named T22-HSNBT-MMAE. This nanoconjugate stands as an excellent candidate for initiating the regulatory preclinical phases prior to phase 1 clinical trials. We firmly believe that this nanoconjugate, selectively targeted to CXCR4+ cells, will be capable of reducing the side effects associated with conventional treatments used in AML. Furthermore, it holds the potential to enhance patient survival by increasing effectiveness and decreasing recurrences linked to the overexpression of CXCR4 in leukemic cells.

2. Results

1. **Generation of a new humanized nanoconjugate:** The main objective of the project has been to generate a novel humanized nanoconjugate, derived from the prototype T22-GFP-MMAE that maintains the same structure and activity but lacks the GFP domain to prevent immunogenicity. Initially, three nanoconjugates containing different domains derived from human proteins were generated and evaluated both in vivo and in vitro. In these nanoconjugates, the GFP domain was replaced with three distinct protein domains: Stefin A (T22-STM-MMAE), human chorionic gonadotropin (T22-CTP-MMAE), and human nidogen (T22-HSNBT-MMAE). Detailed results are described in an article published in 2022 (Serna et al., 2022). Based on the obtained data, particularly the in vivo activity and the required production methodology, we decided to proceed with the development of the nidogen-based nanoconjugate (T22-HSNBT-MMAE) as a humanized replacement for GFP. Additionally, several structural modifications were implemented in the nanoconjugate design, as well as in the methodology for conjugating the toxin MMAE. These modifications have led to improvements in the production and characteristics of the nanoconjugate, enhancing its effectiveness both in vitro and in vivo. On one hand, the linker used in the prototype for conjugating the nanoparticle to MMAE was modified, demonstrating that the same nanoconjugate with a cleavable linker is significantly more potent than when a non-cleavable linker is used. On the other hand, a methodology for site-directed MMAE conjugation has also been refined, allowing precise control over the drug's binding site to the nanoparticle.

2. **Evaluation of in vitro and in vivo antineoplastic activity of T22-HSNBT-MMAE:** With this definitive candidate, several in vitro assays were conducted to assess its mechanism of action in greater detail. Hoechst staining determined that the nanoconjugate induces strong mitotic catastrophe and apoptosis in vitro. Flow cytometry was employed to study cell cycle regulation by the nanoconjugate, revealing an early G2/M cell cycle arrest. Additionally, levels of the DNA damage marker γH2AX and activation of procaspase-3 were quantified, demonstrating that both markers increased in leukemic cells treated with the nanoconjugate. All these results align with the mechanism of action described for MMAE. Furthermore, various in vivo studies were performed using a disseminated AML murine model to demonstrate the antileukemic activity of T22-HSNBT-MMAE. The animal model was generated by

intravenously injecting the human AML cell line THP1. Prior to this, we had transfected this cell line with the luciferase gene, allowing us to track in vivo cell dissemination using the IVIS Spectrum equipment, which measures the bioluminescence emitted by cells in vivo. Utilizing this model, several preliminary studies were conducted to determine appropriate doses that would yield activity without toxicity. It was established that a daily dose of 5 mg/kg of T22-HSNBT-MMAE for 8 days resulted in a complete blockade of leukemic dissemination, nearly tripling the survival of mice (from 15 days to 35-45 days). These results are depicted in Figure 1A (bioluminescence levels) and 1B (Kaplan-Meier survival curves). In all in vivo assays, a comprehensive evaluation of the potential toxicity of the nanoconjugate was conducted. This assessment included complete blood counts, biochemical analyses of liver and kidney function, and histopathological studies using hematoxylin and eosin staining of all organs and tissues. Remarkably, no toxicity was observed in any of the conducted studies.

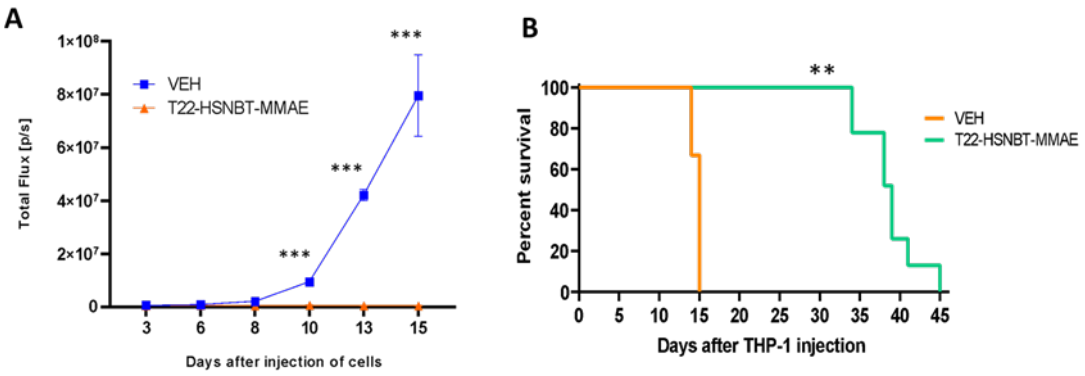

Figure 1. A. Bioluminescence levels during the in vivo experiment using the disseminated AML model with THP1-Luci cells. In the treated group, mice were administered a daily dose of 5 mg/kg of T22-HSNBT-MMAE for 8 days. B. Kaplan-Meier survival curves showing the statistically significant enhancement in survival for the animals treated with T22-HSNBT-MMAE. ** p<0.01; *** p<0,001.

3. Generation of murine models derived from samples of AML patients:

Throughout the project, a total of 136 bone marrow samples were collected from patients at the Hospital de Sant Pau at the time of diagnosis. Among these samples, 92 were ultimately diagnosed with AML. All samples were cryopreserved and utilized for subsequent in vitro and in vivo studies. A comprehensive database was generated, containing both clinical and molecular information of the patients. The CXCR4 levels of the samples were evaluated, establishing a threshold beyond which they are considered CXCR4+. This threshold was determined based on the expression level of

CXCR4 in bone marrow samples from healthy donors. Approximately 50% of the patients were classified as CXCR4+. Notably, the bone marrow samples from healthy donors exhibited very low levels of CXCR4 receptor, as demonstrated by flow cytometry results (Figure 2A) and the representative immunohistochemistry image of CXCR4 (Figure 2B). Figure 2C illustrates the distribution of analyzed patient samples, defining the threshold for determining whether they are CXCR4+ or CXCR4- based on the quantified receptor level in the healthy donor sample.

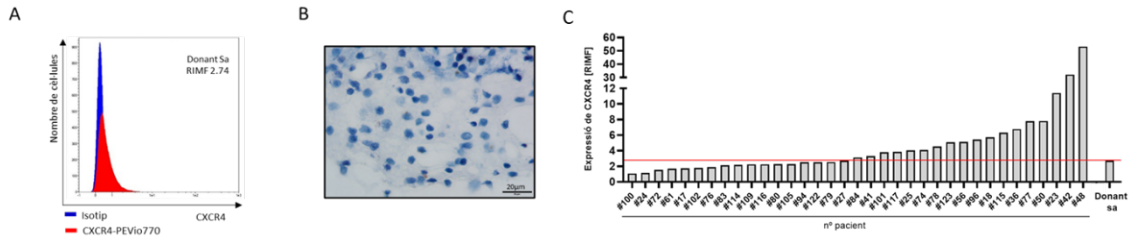

Figure 2. A. Levels of CXCR4, evaluated by flow cytometry, of a bone marrow sample from a healthy donor. B. Representative image of a CXCR4 IHC of a bone marrow sample from a healthy donor. C. Level of CXCR4 expression of some of the AML patient samples and establishment of the positivity threshold based on the levels of the healthy donor.

Finally, we have evaluated the activity of the selected nanoconjugate T22-HSNBT-MMAE in ex vivo samples from AML patients. To determine the dependence on the CXCR4 receptor in the antitumor activity of the nanoconjugate, we used samples from CXCR4+ AML patients (#133, #151, #137), as well as samples from patients who do not express the receptor (#126) and a healthy donor bone marrow. In Figure 3A, the CXCR4 levels of the samples are shown, along with the threshold for considering them CXCR4+ or CXCR4-. Figure 3B displays the results of the ex vivo cell viability assessment, revealing that CXCR4- samples are not sensitive to the nanoconjugate, while CXCR4+ samples exhibit a significant decrease in viability when treated with the nanoconjugate for 48 hours. Thus, the threshold we established based on receptor expression in healthy donor bone marrow samples serves to determine which patients may be sensitive or resistant to the nanoconjugate.

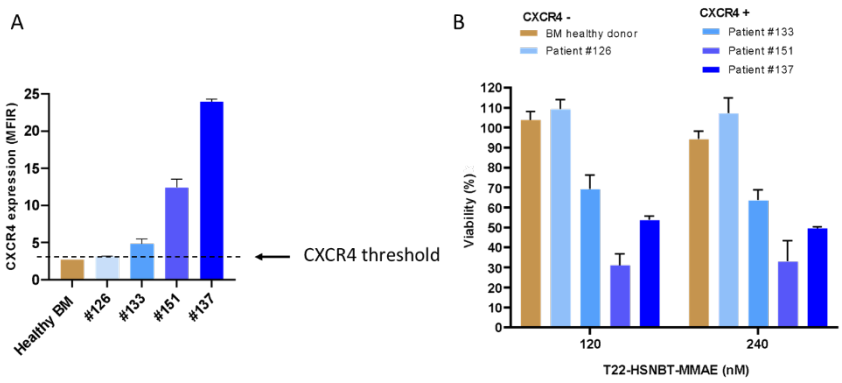

Figure 3. Analysis of T22-HSNBT-MMAE nanoconjugate activity in ex vivo AML patient samples. A. CXCR4 expression levels in bone marrow samples from AML patients and a healthy donor, quantified by flow cytometry. B. Cell viability assays in the previously described samples, evaluating the effect of the nanoconjugate after 48 hours of treatment at 120 nM and 240 nM.

These results are pending validation in vivo using various murine models derived from patient samples (patient-derived xenografts, PDXs) that we have developed during the project. Throughout the project, a total of 8 PDX models have been generated from patient samples with different levels of CXCR4 expression. In all these models, we have determined the post-injection week at which we detect human CD45+ cells in peripheral blood, assessed animal survival, measured the take-rate percentage, and conducted a comprehensive study of all organs and tissues at the end of the study. Finally, we have validated that the human leukemia cells isolated from the bone marrow of mice maintain the same genetic alterations present in the patients from whom they originated.

4. Generation of humanized murine models and evaluation of T22-HSNBT-MMAE activity: In this project, we have optimized two methodologies for generating humanized murine models. These models utilize human T lymphocytes obtained from PBMCs (peripheral blood mononuclear cells) or CD34+ cells derived from umbilical cord samples. For the humanized murine models with T lymphocytes, the most successful humanization results were achieved when starting from a pool of PBMCs from multiple donors. Humanization is considered successful when a minimum of 20% of circulating human CD45+ cells is detected in peripheral blood. In this model, humanization was achieved starting from 20 days post-injection, observed in over 90% of the animals. Additionally, in various experiments conducted, the occurrence of graft-versus-host reaction was very rare, affecting less than 5% of the animals. The primary limitation of this model is its short experimental window, as mice must be sacrificed within a

maximum of 40 days post-injection. For this reason, we have chosen the humanized model generated with CD34+ cells from umbilical cord for evaluating the nanoconjugate. To create this model, we used 4-week-old NSG mice, which were previously conditioned with busulfan, and then injected them with a total of 1-2 × 10^5 cells from commercial umbilical cord samples. Between 10-15 weeks post-injection, depending on the donor, we already observed the presence of human CD45+ cells in peripheral blood. By 15-18 weeks, we also observed the appearance of CD4+ and CD8+ T lymphocytes. At this point, we determined that it was the most suitable time to inject bioluminescent THP1 cells to generate the humanized murine leukemia model for evaluating the effect of the T22-HSNBT-MMAE nanoconjugate. Once leukemia was established, we analyzed the nanoconjugate's antitumor effect, confirming that its antitumor activity remained unaltered and that there were no relevant toxicities or adverse effects.

3. Relevance and possible future implications

The primary clinical implication of the results obtained in this project lies in the incorporation of a new treatment for AML patients who overexpress the CXCR4 receptor. After completing the project, we are continuing the development of the nanoconjugate in collaboration with the company Nanoligent, which holds the patent. Following the completion of all relevant regulatory studies, we plan to initiate a phase 1 clinical trial. This trial will include patients not only with AML but also with other cancer types that exhibit high levels of CXCR4. Thus, the intention is to conduct an agnostic tumor phase 1 clinical trial, selecting patients based on CXCR4 receptor expression. The potential clinical implications of this project extend beyond AML treatment, as this nanoconjugate could also be applied to other tumor types.

The approach proposed in the development of the nanoconjugate obtained in this project is novel and promising for several reasons:

- The **specific targeting** of a drug to CXCR4+ cells promises an improvement in treatment efficacy, as it allows for increased drug concentration in tumor cells while reducing toxicity by significantly decreasing its distribution in normal tissues.

- The chosen **target receptor** will not only allow for this therapeutic approach in patients with different types of cancer, but it is specifically aimed at those patients who are less likely to respond to conventional therapy and have a worse prognosis. The CXCR4 receptor has been associated with poor prognosis, increased metastatic capacity, chemoresistance, and a higher risk of relapse in various solid and hematological tumors. Therefore, we are developing an antitumor therapy specifically focused on patients with poor prognosis.

- **Antimetastatic effect:** In most tumor types, metastases are the primary cause of death, with the primary tumor generally being more manageable through surgery or radio/chemotherapy. Given the strong association between CXCR4 expression and the metastatic capacity of tumor cells, we can consider that the nanoconjugate developed here could also be effective in blocking metastases in solid tumors, as observed in the significant reduction of leukemia dissemination.

- **Social impact:** The development and regulatory approval of an antitumoral nanoconjugate for AML would not only have a direct clinical impact but could also significantly affect society. This innovation could lead to an improvement in the quality of life for patients and their families, as well as a reduction in the costs associated with AML treatment and complication management.

4. Generated and scientific bibliography

Scientific publications

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Doctoral Theses

Title: Simple biochemistry for complex protein materials.
Doctoral student: Héctor López Laguna
Doctoral Program: Biotechnology (Universitat Autònoma de Barcelona)
Defense Date: 31/03/2023

Title: Nanoparticles targeted to CXCR4+ stem cells for the treatment of acute myeloid leukemia.
Doctoral student: Yaiza Núñez Amela
Doctoral Program: Biomedicine (Universitat de Barcelona)
Defense Date: 12/04/2023

Title: Exploring and exploiting multi-domain recombinant proteins as targeted nanomedical tools.
Doctoral student: Eric Voltà Durán
Doctoral Program: Biotecnologia (Universitat Autònoma de Barcelona)
Defense Date: 12/09/2023

Title: Preclinical development of humanized nanoconjugates selectively delivered to CXCR4+ cells in murine models of acute myeloid leukemia.
Doctoral student: Annabel Garcia León
Doctoral Program: Biomedicina (Universitat de Barcelona)
Start Date: September 2021