Research Project

Synthesis of a Venetoclax-Lysozyme drug conjugate: a kidney regenerative therapy

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Abstract:

Introduction: The cells of our bodies can enter a senescent state in which they lose their ability to grow and divide but they do not enter apoptosis. The accumulation of senescent cells contributes to inflammation and age-related diseases. In the kidney, their accumulation causes kidney diseases. The elimination of senescent cells enhances regeneration and improve tissue functionality. Venetoclax is a BCL-2 inhibitor used in cancer but has also shown a good senolytic profile. Nonetheless, Venetoclax does not accumulate in kidney and its administration as senolytic agent would cause severe adverse reactions. Synthetising a drug conjugate to deliver Venetoclax to kidney can regenerate kidney functionality and minimize the risk of adverse reactions.

Methods and results: We attempted to direct Venetoclax to kidneys using chicken egg white lysozyme bounded through Platinum linkage technology. Due to solubility incompatibilities, the lysozyme was replaced by PAMAMA G3 dendrimer. The identity and drug release capability were assessed through displacement reactions with KSCN.

Conclusion: UPLC and displacement reactions indicate the conjugation of Venetoclax-Lx-Dendrimer was successful, further tests must be performed to confirm safety and efficacy of the conjugate in kidney cells. To account for possible accumulation problems, we suggest repeating the synthesis using a biodegradable dendrimer instead.

Layman's Summary

Kidneys can lose their function and structure due to age and other diseases. Through time kidneys accumulate old cells that do not function properly anymore but do not enter cellular death. These cells are called senescent cells. In addition to their diminished functionality, these cells secrete a lot of small proteins and components that cause inflammation to neighbouring cells, and this makes the whole organ sick.

Causing the death of senescent cells allows the kidney to regenerate with new fully functional cells. This benefit has been observed in animal studies.

Drugs used for curing cancer are studied for killing senescent cells, a good candidate is Venetoclax (VNTO) a drug that is used for leukaemia. Despite this drug seems promising in several studies, the drug does not reach the kidneys and can cause a lot of adverse reactions systemically.

In this study, we attempt to make a delivery system to direct VNTO to the kidneys with the purpose of reducing adverse reaction and improving the senescent action of the drug directly in the desired organ.

The synthesis of such drug conjugate has 3 components, VNTO which is the active agent, a carrier which is the component that will direct the drug to the kidney and a linker to

bring the previously mentioned 2 components together. This is necessary because VNTO does not have chemical groups to react directly with the carrier.

The synthesis was attempted using chicken egg white lysozyme (LZM) as the carrier system and a Platinum II as the linker (Lx). First VNTO was linked to Lx. Consequently, LZM was modified to add methionine groups in their lysine terminals, this would also improve the linkage capacity between the components. Finally, the modified LZM was reacted with the VNTO-Lx to form the final conjugate.

The main benefit of using LZM is that it's not toxic and does not accumulate in the body. The Lx has already been used to link drugs that do not have active groups to bind directly to the carrier and has shown to be able to release the drug.

In addition, both LZM and Lx have been successfully used in previous synthesis to direct drugs to the kidneys showing to cause no adverse effects. Nonetheless, during the synthesis with LZM, a big difference in the solubility between LZM and VNTO made the synthesis impossible.

Multiple tests were carried out to determine whether it was possible to carry out this final step in a proportion of organic solvent (DMF) and water that would not cause precipitation of any of the components of the carrier system. Yet the solubility differences were incompatible for this synthesis.

The solubility problems forced us to select a different carrier system, a dendrimer. A dendrimer is a branched polymeric structure in which new components can be added at the end of each branch or even encapsulated inside the dendrimer pockets. VNTO was first linked to the Lx and consequently to the dendrimer. The formation of a new compound after this reaction was evident using UPLC, the newly formed species were evident.

Through a reaction to displace VNTO from the conjugate, it was possible to confirm the compound can release the drug. This reaction also helps as confirmation that the new peak was the conjugate of our interest. However, to confirm more details about the formed compound such as the proportion of VNTO linked per dendrimer, more precise laboratory techniques like mass spectrometry must be used.

Due to time limitations, it was also not possible to confirm the drug identity with Maldi ToF and it was not possible to test its safety and efficacy in vitro.

By the end of this research project, evidence points that a conjugate was formed between VNTO and the dendrimer which is a promising therapy for KDs. It was also possible to confirm the formed conjugate can release the load by competition. It is recommended at this point to continue with characterization test and purification techniques to be able to continue with in vitro models.

A recomendation is made to replace the dendrimer by a organic dendrimer to account for probable accumulation of the non degradable dendrimer in kidney cells.

Introduction

A variety of diseases can cause kidneys to lose their function or structure over time causing kidney diseases (KD) such as chronic kidney disease (CKD) or Acute Kidney disease (AKI). The main difference between these diseases is that CKD can take decades before the symptoms are evident whereas AKI has a very fast onset (1–4). Despite any differences, there are mutually reinforcing interactions between AKI and CKD; Patients who develop AKI and don't have a complete recovery, can progress into CKD. At the same time, patients who suffer from a progressive and chronic loss of kidneys function and structure can fall into AKI (5,6).

Available treatments can prolong the progression of the disease, but to date there is no cure for KD. Current treatments attempt to control signs and symptoms; antihypertensives, diuretics, erythropoietin, iron, statins, calcium and vitamin D supplements together with a strict diet are part of treatment guidelines. Dialysis is required in later stages, until a compatible donor is found, yet kidney transplantation success is uncertain (3,4).

The most common risk factors for KD are age, diabetes, hypertension, cardiac disease and glomerulonephritis. Elderly patients have a higher risk to develop KD as well as to develop the risk factors that can lead to KD. With age, there is a progressive loss in renal mass, decrease in renal growth factors, decrease in renal blood flow and presence of glomerulosclerosis, all leading to a decrease in the glomerular filtration rate (GFR) an important marker for KD (6). Therefore, the elderly are the most vulnerable population for developing KD.

Currently, over an 11% of the world population is over 65 years of age and this number is expected to double by 2050 (accounting for 2 billion people). The increase in the global elderly population, will consequently increase the incidence, mortality, morbidity and economic and social impact of KD (7).

The economic burden of kidney diseases is considerably high, especially when dialysis is required. The costs vary per country and per stage of the disease. In the United States for example, the treatment of patients in stage 4, 5 or end stage (ESKD) goes up to $53 000 yearly. Nevertheless, the real cost of KD is higher when other factors such as social impact, loss of productivity, domestic costs and inability to participate in community activities are considered (8,9).

Due to the huge economic and social impact KDs have and considering that the elderly population will continue to grow, it is of enormous importance to find solutions to KDs.

Research has found that many age-related diseases, including KDs, are related to the accumulation of senescent cells. Senescent cells are cells that have accumulated some forms of damage for example, small genetic damage, oxidative stress, mitochondrial disfunction or shortening of telomers, and that despite this damage, they do not enter

apoptosis but enter a phenotype of proliferation arrest (10,11). These cells show morphological and structural changes that impair homeostatic and regenerative mechanisms (12–15).

On top of their limited functionality, senescent cells have a particular secretome phenotype known as senescence associated secretory phenotype (SASP) which is characterized by the presence of several inflammation markers, inhibition of stem cell function and remodelling of extracellular matrix which limits tissue regeneration and creates a disfavouring environment for neighbouring cells (16).

The removal of senescent cells or senolysis improves organ function, causing a healthier and longer lifespan. This benefit was observed by Baker et al (17) in mice models in kidney, heart and fat tissues.

Navitoclax, Venetoclax and Dasatinib are cytotoxic agents that also cause apoptosis of senescent cells (18,19). Despite being very efficient in this task, they are unspecific, which causes cellular death in other tissues where the effect is not desired and severe adverse reactions such as neutropenia, diarrhoea, nausea, fatigue, anaemia and thrombocytopenia. Developing a kidney targeted delivery system would allow to obtain the senolytic effect in these organs while decreasing systemic adverse reactions.

Dasatinib in combination with quercetin act as senolytic agent by altering the level of klotho/Wnt/β-catenin and is already in clinical trials (20,21). While Navitoclax and Venetoclax bring the BCL proteins in the cell into balance and this leads to cellular death. The development of a kidney targeted senolytic agent with Navitoclax was already attempted in the Pharmaceutics department of Utrecht University, yet the results of that synthesis pointed at solubility incompatibilities and low yields.

The small chemical differences between Navitoclax and Venetoclax could imply important differences in the synthesis of the compound which makes it worth trying to develop a kidney targeted senolytic agent using Venetoclax as the active agent.

To develop a kidney targeted delivery system, it is necessary to observe the anatomical and physiological characteristics of the target site to make a conclusion of what would be the best place to target.

Proximal tubular cells play a very important role in kidney functionality, they oversee the reabsorption of two thirds of the filtrate volume and several components from urine. Consequently, delivering a senolytic therapy to these cells should cause the greatest regenerative impact.

To reach proximal tubular cells, the drug conjugate first needs to pass through the Glomerular Endothelial Cells (GEC), where the fenestrations have a size of 60-160nm. Then it must pass through the Glomerular Basement Membrane (GBM) where the mesh is about 4-10nm. GBM is made mostly of heparin sulphate proteoglycan that provides this membrane with a high negative charge. This charge repels negative charged particles and facilitate the passage of positively charged molecules. To pass this barrier the

conjugate has to observe the mesh size and be neutral to positively charged. The last barrier to overcome is that of the podocytes, where the slit pores are about 30-40nm (22,23).

In addition to the physiological considerations, the components of the synthesis must observe certain chemical compatibility to effectively bind together.

The success of a targeted therapy strives in the selection of the appropriate components. There are 2 main components in this synthesis, first the therapeutic agent and second the carrier agent. The rationale on the selection of the components for this synthesis will be further explained bellow.

BCL2 Inhibitors

BCL-2 is a family of proteins that regulate the survival fate of cells. The members of this family can be classified as pro-apoptotic (Bak, Bax, Bad,Bid, Bim, Bmf, Novax, Puma) or anti-apoptotic (BCL-2, BCL-xl, Mcl-1/2, Bcl-w, Bd-b and Bfl-1/A1). The balance between these 2 contradictory members determines whether the cell enters apoptosis or resists it. (19,24).

BCL2 family of protein exhibit sections of homology called BH domains. The biochemical difference between pro and anti-apoptotic is that pro apoptotic BCL-2s only have a BH3 domain that is essential for the apoptotic effect (19,24,25).

In normal conditions, anti-apoptotic proteins sequester pro-apoptotic members and maintain the mitochondrial barrier integrity, but when the balance leans towards apoptosis, the oligomerization of Bak and Bax causes mitochondrial outer membrane permeabilization and the consequent activation of the caspase pathway leading to apoptosis as shown in Figure 1 (19,24).

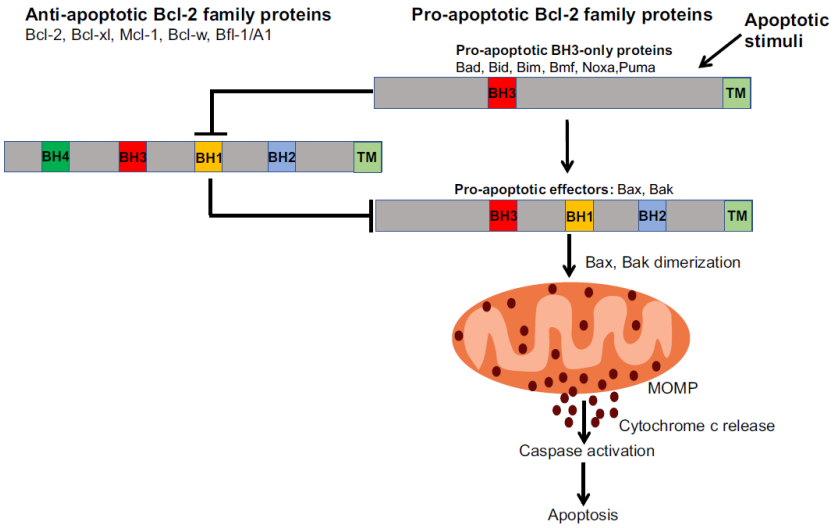

Figure 1. BCL-2 proteins family and apoptosis mechanism(19).

Antiapoptotic BCL2s are highly upregulated in several cancer types. This makes BCL2 inhibitors particularly effective to treat lymphoma, leukaemia and some solid tumours (25). Similarly, some senescent cells can inhibit apoptosis by expressing higher levels of anti-apoptotic BCL-2 proteins. In fact, apoptosis resistance has been confirmed in diverse senescent cell types (19,26).

It is important to highlight that in addition to their antineoplastic effect, BCL-2 inhibitors have also shown senolytic activity in senescent HUVECs and IMR90 cells and in animal studies. The clearance of senescent cells with BCL2 inhibitors has demonstrated to improve neurovascular coupling in aged mice (27,28).

There are to date 2 BCL-2 inhibitors approved for clinical use. Venetoclax and Navitoclax. It could be argued that the BCL-2 inhibitor with the broader inhibition spectrum will provide a more potent senolytic profile (29). While Venetoclax is highly sensitive and more potent to inhibit BCL-2, Navitoclax is 200-fold more potent as BCL-XL inhibitor and can also inhibit BCL-W (29,30) Nevertheless, the synthesis of Navitoclax drug conjugate using egg white lysozyme is difficult and provides low yields, as demonstrated by previous research in the Pharmaceutics department of Utrecht University. Seen that Venetoclax also exhibits a good senolytic profile, it is important to explore the feasibility of the synthesis of a Venetoclax drug conjugate and its efficacy as senolytic therapy for kidney diseases.

Venetoclax is the first BCL2 inhibitor approved for the treatment of relapsed or refractory chronic lymphocytic leukaemia with 17p deletion, lymphoma, myeloma (especially those with translocation (11;14) and leukaemia (31). Venetoclax mimics the activity of BH3 domain of pro apoptotic proteins and binds with high selectivity to BCL-2. Its use is considered safe, but it is known to cause thrombocytopenia, neutropenia and anaemia therefore, a target delivery would minimize the probability of these adverse reactions (31).

Venetoclax (Figure 2) represents the therapeutic agent of this synthesis.

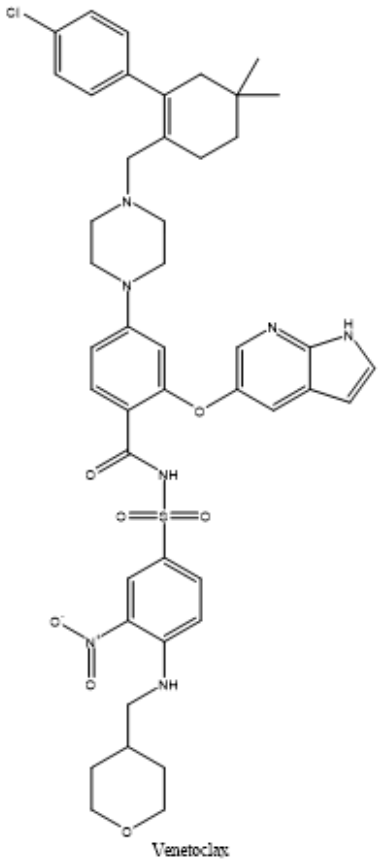

Figure 2. Venetoclax molecular structure.
MW: Venetoclax (868.44 g/mol).

Chicken Egg White Lysozyme

Chicken egg white lysozyme (LZM) is a small weight protein (14.33kDa) with a diameter of approximately 7.9nm (32).

The main advantage of using LZM as a carrier agent is that due to its biological nature, it does not cause toxicity related to accumulation or clearance mechanisms. LZM has been widely studied as a kidney drug target carrier. It has been used for the successful delivery of small molecules such as naproxen, kinase inhibitors and antibiotics (33–35).

LZM is freely filtered through the glomerulus and is reabsorbed in proximal tubule epithelial cells by the membrane receptor megalin (36).

In the presence of the proper chemical groups, it is possible to bind drugs with the carrier through a direct linkage of both components. The most common reversible linkages for drug-carrier conjugates are amide, ester, disulphide, carbamate, carbonate, hydrazone and coordinative bonds(37–39). An interesting example of this direct bonding is that between LZM and naproxen. The naproxen's carboxylic acid group reacts with N-hydroxysuccinimide for an even more reactive form that binds directly to the terminal lysine groups of the LZM as seen on Figure 3 (38).

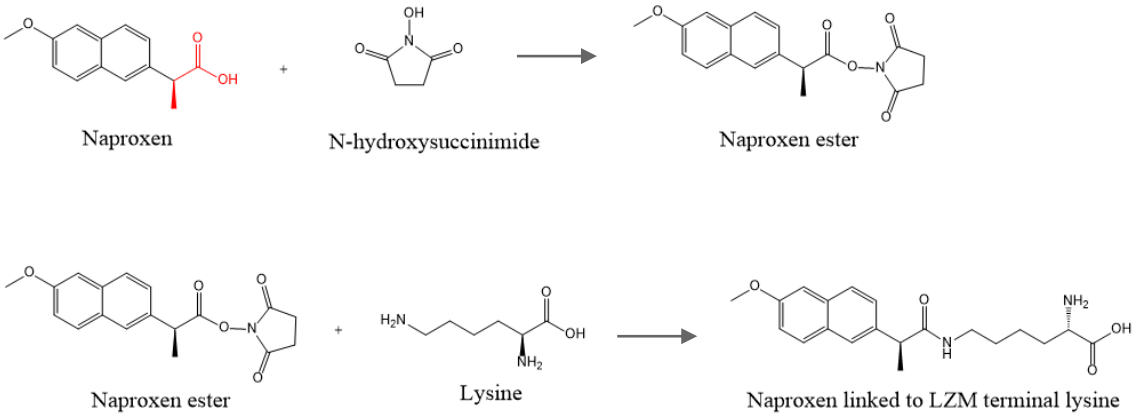

Figure 3. Naproxen covalent reaction to Lysine groups of LZM, Naproxen active carboxylic group highlighted in red (38).
MW: Naproxen (230.26 g/mol), N-hydroxysuccinimide (115.09 g/mol), Naproxen ester (344.35 g/mol), Lysine (146.19 g/mol), Naproxen-Lys (358.442 g/mol).

It is possible to note on Figure 4 that VNTO lacks functional groups such as the carboxylic group present in Naproxen to be able to bond directly to the Lys terminal groups of the LZM. Therefore, The delivery system must include a third agent, a binding agent to link VNTO and LZM together.

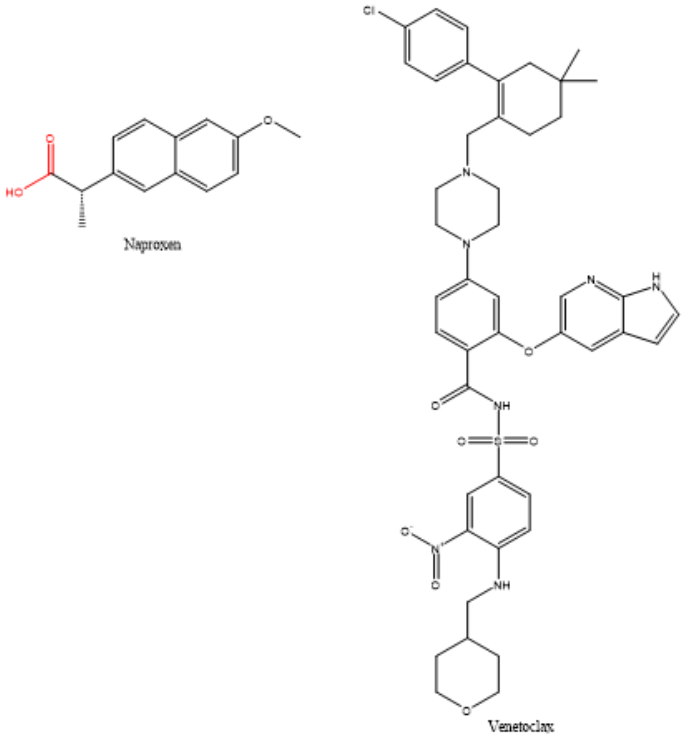

Figure 4. Comparison of Venetoclax and naproxen molecular structures.
MW: Naproxen (230.26 g/mol), Venetoclax (868.44 g/mol)

Universal Linker System ULS™.

Platinum II can bind reversibly through coordination with atoms that can donate electrons, this characteristic has been exploited to link a payload and a carrier that present aromatic nitrogen or sulphur atoms, including proteins. This linkage approach using platinum II is known as Universal Linkage System (ULS™) (41).

It has been observed that drug conjugates using ULS™ release slowly, allowing a sustained release mechanism. The release of the payload from the linker and carrier occurs due to competition with agents that have a higher donor capacity for example glutathione sulphur group.

ULS™ has been successfully used for the delivery of a payload into the kidney using LZM as carrier (33–35,42). This evidence supports that the linking between LZM and the Pt atom of the linker is possible nonetheless, the synthesis of a conjugate using VNTO comes with particular challenges due to the hydrophobic characteristic of this drug.

Although the ULS™ has a similar chemical structure as cis-platinum- the cytotoxic agent used in cancer- once the Lx has been bonded to the drug and the carrier, it is no longer reactive to bind to DNA strands (39). Despite previous evidence of safety of the USL™, it is necessary to study the safety of the final conjugate *in vitro* and *in vivo*.

From here on, The ULS™ will be referred to as “Linker” or “Lx”.

All considerations observed, it is of interest to study the possibility of synthesising a drug conjugate to deliver VNTO to proximal tubular epithelial cells as a senolytic therapy for the treatment of KDs.

Taking the nature of the components into account, this synthesis poses a challenge to determine the most appropriate conditions, but its success could bring hope to millions of people who suffer from KDs around the world.

The aim of this study is to explore the linkage of VNTO to a carrier targeted to kidney cells to cause senolysis and restauration of kidney function. The study also aims to evaluate whether this synthesis is reversible which would indicate the formed conjugate could release the drug in the target site, as well as analysing the toxicity and the effectivity of the final conjugate in kidney cells in vitro.

The chemical structure of the final conjugate we attempt to synthesize with this research is illustrated in Figure 5.

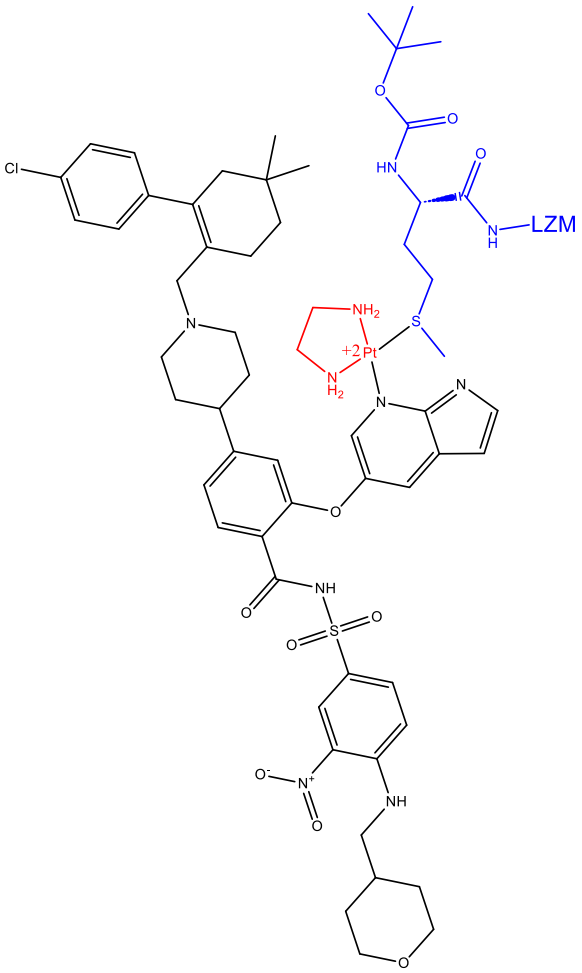

Figure 5. Expected chemical structure of the delivery system aimed to synthetize with this study. VNTO is in black, the Lx is in red and the methionine group of the LZM is in blue.
MW: Drug conjugate 15659.85g/mol

Materials & Methods:

Venetoclax-Pt-LZM conjugate synthesis

The Venetoclax- drug conjugate was carried out following a 4-step synthesis as shown in Figure 6. Briefly described; The first step was the activation of the dichloro(ethylenediamine)platinum II to obtain the linker. The second step was the conjugation of the linker with Venetoclax. In a 3rd reaction, the amino terminals of the Lysozyme were modified to methionine terminal ends and the final step was the conjugation of the activated Venetoclax-Lx with the modified LZM-Met.

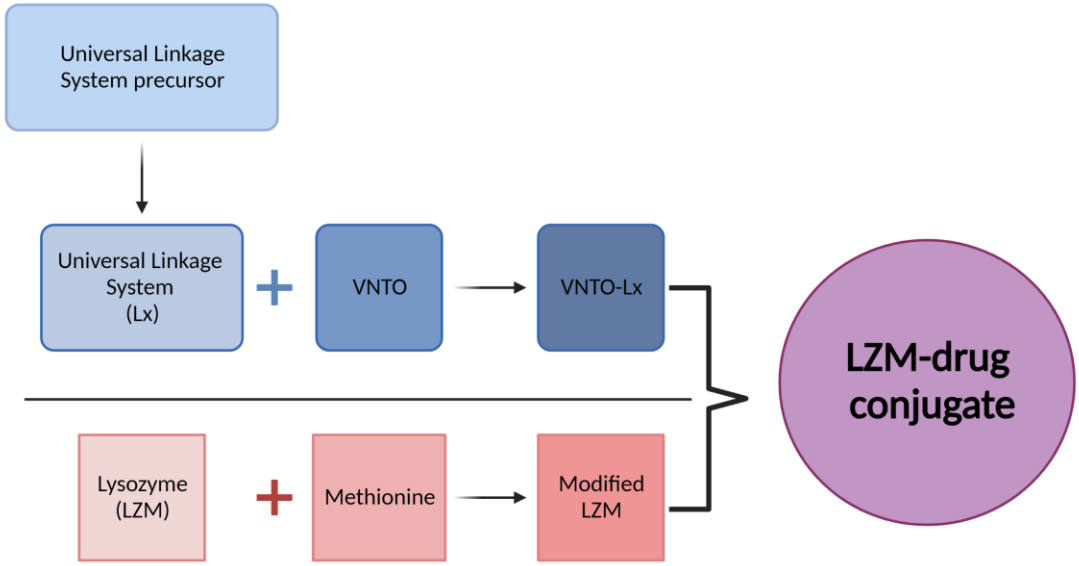

Figure 6. Venetoclax- LZM drug conjugate synthesis diagram.
Note: Chemical structures and reactions schemes are listed in every method section where a reaction takes place.

Step 1. Platinum activation.

The activation of the dichloro(ethylenediamine) platinum II into the reactive form of the linker followed the method described by Sijbrandi et al, 2017 (43). A stock solution of the linker was obtained by incubating 150mg of dichloro(ethylenediamine) platinum II (0.46 mmol) with a molar equivalent amount of $AgNO_3$ (78.14mg) (0.46mmol) in 5mL of dimethylformamide (DMF). The mixture was reacted in the dark during 16 hrs. The reaction product was then filtered through Celite® to remove the AgCl precipitate, 3mL extra of DMF were used to flush the filter, the total volume of the reactive form of the linker was 8ml. Figure 7 shows the chemical activation of dichloro(ethylenediamine) platinum II.

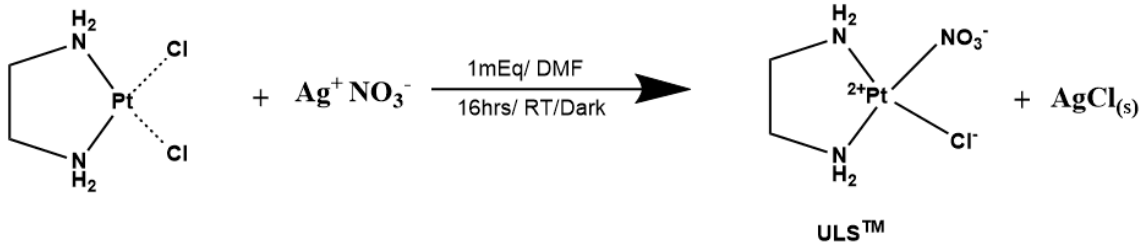

Figure 7. Platinum II activation reaction.
MW: Dichloro(ethylenediamine) platinum II (326.08 g/mol), Lx mono chloro mono nitrate (352.63 g/mol), Silver nitrate (169.87 g/mol).

Step 2. Venetoclax- platinum conjugation.

To determine the most appropriate setup for the reaction with Venetoclax, 2 small scale synthesis were carried out. The reaction was expected to occur as shown on Figure 8.

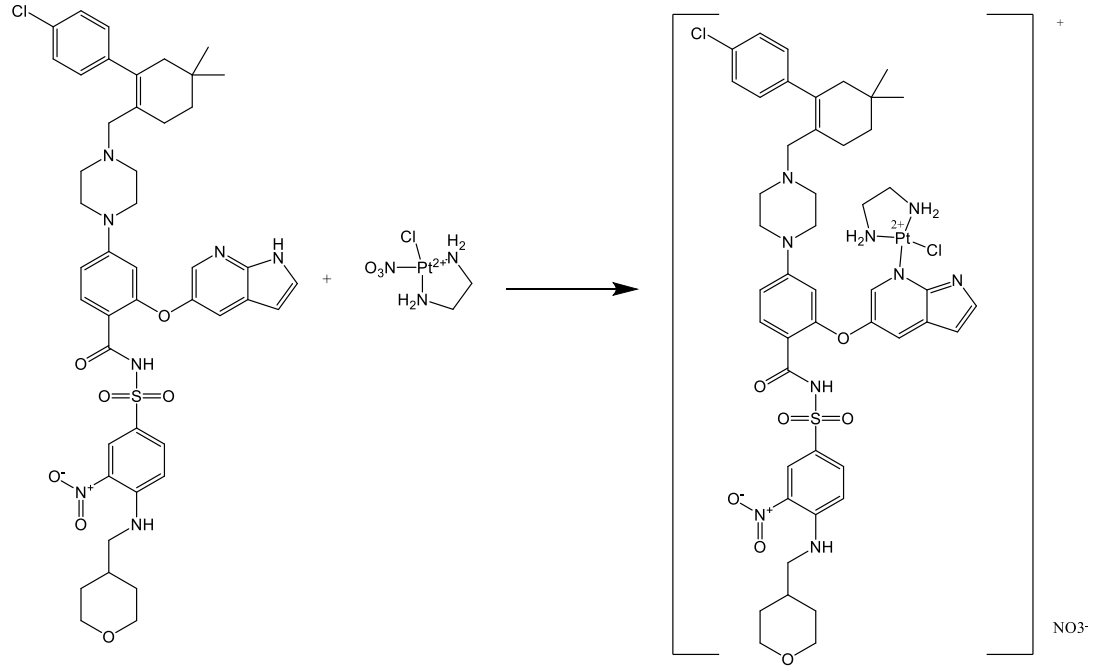

Figure 8. Possible bonding between VNTO and the Linker

MW: VNTO (868,44 g/mol), Lx mono chloride mononitrate (352.63 g/mol) [VNTO-Lx] (1158.32 g/mol).

Test synthesis 1. Reaction with molar equivalent mounts of VNTO and Lx at room temperature.

For this test 7 mg of VNTO (8µmol) were reacted with 2.84mg (8µmol) of Lx in 1ml DMF. This reaction with molar equivalent amounts of both reagents was carried out in the dark, at room temperature with magnetic stirring and monitored during 6 hours with samples taken at 1,2, 4 and 6 hrs.

Test synthesis 2. Reaction with molar equivalent amounts of VNTO and Lx at 60°C.

For this test 7 mg of VNTO (8µmol) were reacted with 2.84mg (8µmol) of Lx in 1ml DMF. This reaction with molar equivalent amounts of both reagents was carried out at 60°C in the dark, with magnetic stirring and monitored during 6 hours with samples taken at 1,2, 4 and 6 hrs.

Displacement of VNTO with KSCN.

Three solutions of KSCN in DMF were prepared and incubated with VNTO-Lx at 60°C. KSCN was added in 10, 100 and 1000 fold molar excess, the reaction was carried out in the dark, with magnetic stirring. The amounts of reagents used is shown on Table I. The reaction was real time monitored in UPLC taking samples at 1,2,4,6 and 24 hrs of reaction. Figure 9 shows the displacement reaction.

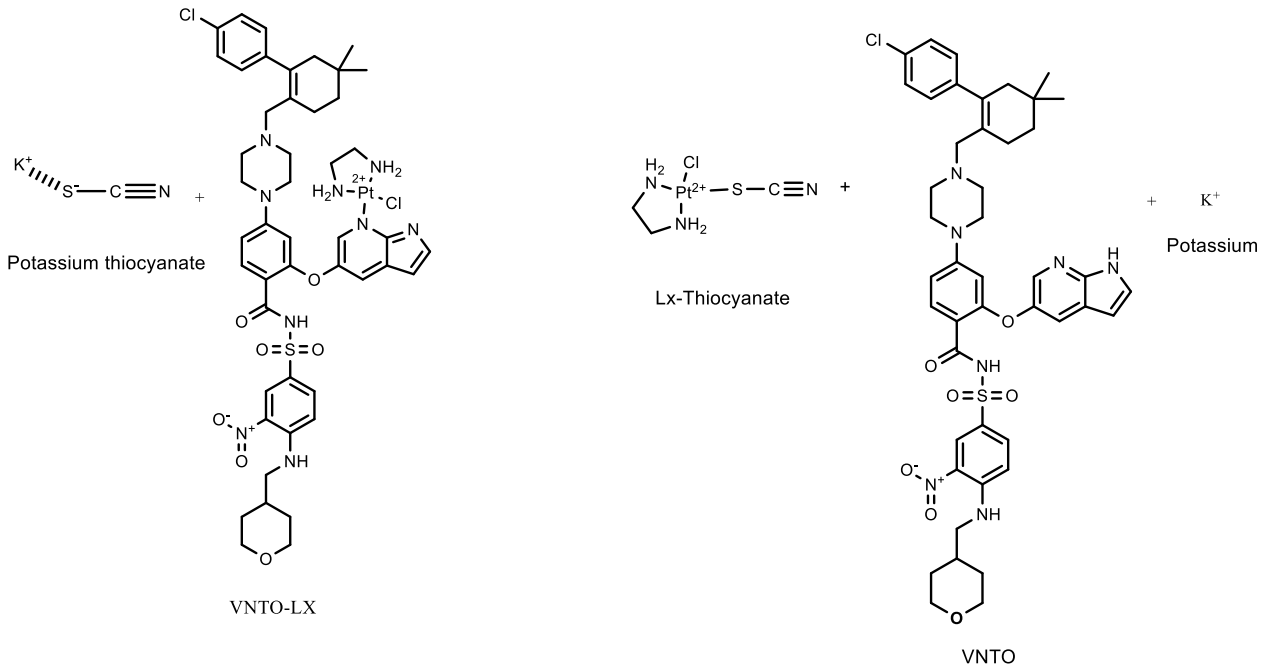

Figure 9. Displacement of VNT by potassium thiocyanate.
MW: Potassium thiocyanate (97.18g/mol), VNTO-Lx (1158.07 g/mol), Lx-thiocyanate (348.71 g/mol).

Table I. Excess of potassium thiocyanate for VNTO displacement from VNTO-Lx.

VNTO : KSCN (mol : mol)	**VNTO-Lx (mg)**	**KSCN (mg)**	**DMF (mL)**
1:10	1.159mg	0.9718	1
1:100		9.7180	
1:1000		97.1800	

Step 3. Lysozyme -Methionine modification.

LZM was modified to expose methionyl groups as described by Fretz (44). The reaction can be seen in Figure 10. Briefly explained; 200mg LZM (13.96µmol) were dissolved in 20ml PBS buffer and were incubated with 14.55mg of Boc-Met-Osu (42.00µmol) contained in 0.5ml DMSO. The mixture was stirred for 1 hr at room temperature. The product was dialyzed using a cellulose tube dialysis filter with a molecular weight cut off of 3500Da against water for 48 hrs. The water was changed 2 times per day. The product was filtered through a 0.22um RC filter, then it was freeze dried overnight. The resulting powder was stored at -20C.

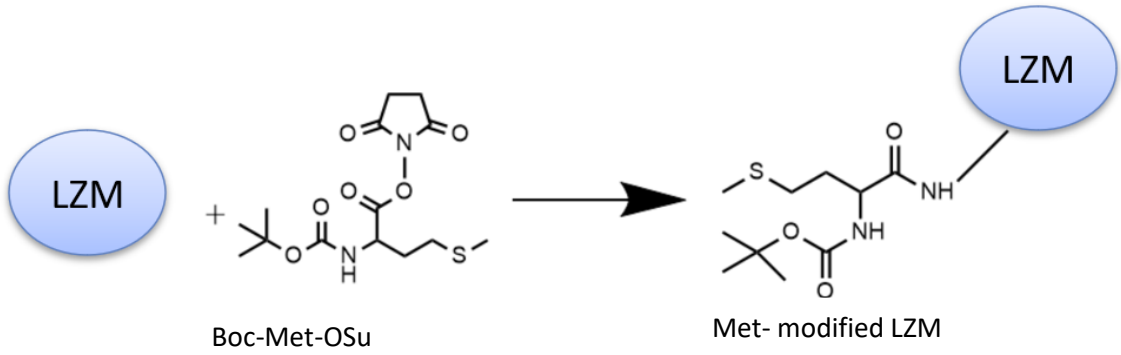

Figure 10. Methionine modification of LZM. One methionine adhesion to LZM. The methionyl is attached to the lysine side chain amino group.
LZM: 14305 g/mol, Boc-Met-OSu: 346.40 g/mol, LZM-Met: 14537 g/mol.

3.1 Lysozyme fluorescent labelling staining

The labelling was performed by reacting 100mg of LZM (6.98µmol) dissolved in 10mL of PBS and 8mg of a FITC (20.55µmol) contained in 0.5ml PBS. The FICT solution was added drop wise to the LZM. The reaction occurred during one hr at room temperature with magnetic stirring. The product was dialyzed against PBS during 48hrs for its consequent overnight lyophilization and finally stored at -20C.

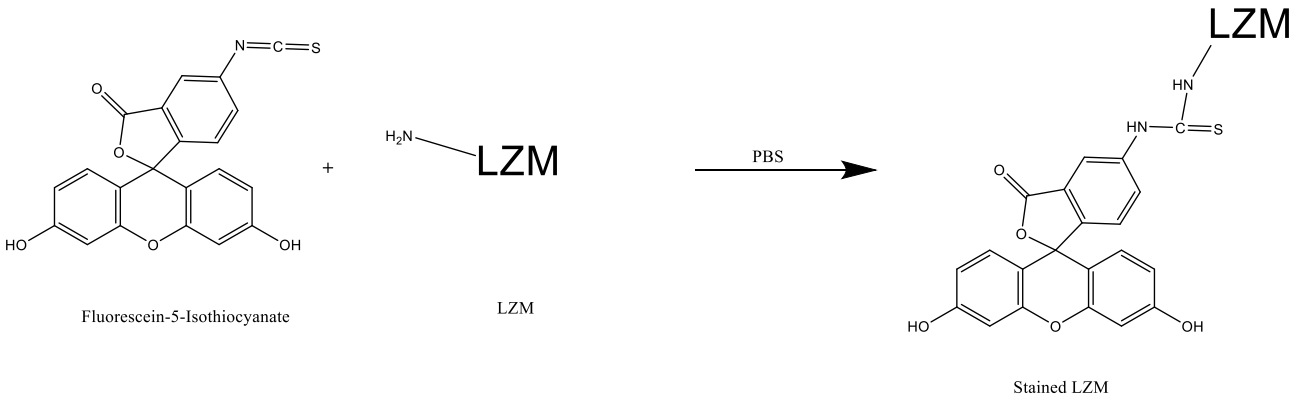

Figure 11. Fluorescent labelling of LZM.
MW: FICT (389.38 g/mol), LZM (14.33KDa), Stained LZM (14719 g/mol).

Step 4. Venetoclax-platinum conjugation to LZM-MET.

This step brings together all products of the previous reactions into the final drug conjugate. The main challenge for this reaction was the solubility characteristics of both species. Yet, the first conjugation attempt was carried out without any solubility considerations as follows. Figure 5 shows the expected structure of the conjugate.

First conjugation attempt:
2.7mg VNTO-Lx (2.5umol) contained in 250µl of DMF with 18.4mg LZM-Met (1.25umol) contained in 3ml PBS. This reaction has a molar proportion of 2VNTO-Lx : 1LZM-met. VNTO-Lx was slowly added to the LZM-Met The mixture was stirred overnight in the dark at room temperature. The mixture was then filtered using a 0.22µm RC filter. The final product was protected from light and stored at 8°C.

Dissolve LZM in water instead of PBS:
LZM-Met is dissolved in PBS, the effect of this salts was studied by repeating a conjugation attempt but this time using water instead of PBS as solvent for LZM-Met. In addition, the molar amount of VNTO-Lx was reduced by reacting to 1VNTO-Lx: 1LZM-Met.

10.39mg of LZM (0.699µmol) were dissolved in 2.965mL of water. 0.61mg VNTO-Lx (0.7µmol) dissolved in 35µL DMF were added slowly. The mixture was stirred overnight in the dark at room temperature. The mixture was filtered using a 0.22µm RC filter. The final product was protected from light and stored at 8°C.

Use acidified water:
Since acidifying the water can improve VNTO's solubility, the reaction was repeated using acidified water at pH5 as follows: 10.00mg LZM (0.673µmol) were dissolved in 2.965mL of Acetate Buffer pH=5. 0.61mg of VNTO-Lx (0.7µmol) contained in 35µL DMF were added slowly. The mixture was stirred overnight in the dark at room temperature. The mixture was filtered using a 0.22µm RC filter. The final product was protected from light and stored at 8°C.

Solubility Tests.

The results of the conjugation between VNTO-Lx and LZM, were not promising. Solubility tests were carried out as follows:

VNTO-Lx Solubility:
A stock solution of 2.0µmol/ml (1.74mg/ml) VNTO-Lx in DMF was used for this test.
100µL of this solution were dissolved in different proportions of organic solvent/water. In other words, 0.2µmol of VNTO-Lx (0.2317mg) were dissolved in different proportions of DMF/water (10/90, 20/80, 30/70, 40/60, 50/50 and 60/40).
The solutions were centrifugated 1200rpm for 2 minutes and the concentration of VNTO-Lx in the supernatant was determined with UPLC.
The test was repeated using DMA instead of DMF.

LZM-met Solubility:
A stock solution of 1.02µmol/ml (15.00mg/ml) LZM-FICT in water was used for this test.
100µL of this solution were dissolved in different proportions of organic solvent/water.

In other words, 0.10µmol LZM-FICT (1.5mg) were dissolved in different proportions of DMF/water (10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, 80/20 and 90/10.
The solutions were centrifugated 1200rpm during 2 min and the precipitation was visible at the naked eye. In the cases where the precipitation was not evident, agglomeration and precipitation were confirmed using DLS.
The test was repeated using DMA instead of DMF.

Materials

Dichloro(etylendiamine) Platinum II 99%, fluorescein-5-Isothiocyanate (FICT) >90%, silver nitrate >99%, potassium thiocyanate >99%, PAMAM dendrimer ethylenediamine core generation 3 solution 20%wt in MetOH, Celite® 545 and the chicken egg white lysozyme 90% > 40000 unit/mg were obtained from Sigma-Aldrich, Venetoclax >99% was bought from Pub Chem Express and the Boc-Met-OSu from Bachem. The solvents acetonitrile >99%, dimethylformamide >99%, dimethylacetamide >99% and dimethyl sulfoxide >99%were supplied from Biosolve.

Characterization Methods

UPLC

Method: 30_100B dual WL 220_318.
UPLC characterization was carried out using a Waters Acquity ™ Ultra Performance LC with a Waters UPLC BEH C18 (1.7µm 2.1 x 50mm) column; mobile phase A= ACN 30% + 0.1%FA, mobile phase B=ACN 100%+ 0.1%FA; strong needle wash= 70/20/10 MetOH/H_2O/IPA; weak needle wash ACN 30% pump flow= 0.4mL/min; injection volume = 5µL; column temperature 50C; sample temperature= ; detection wave length= 318; gradient: t=0min 0% B; t=1min 0%B; t= 4 min 20%B; t=6min 60%B; t=9min=100%B t=10min 0%B; requilibration= 3min.

Method: 40_100B dual WL 220_318
UPLC characterization was carried out using a Waters Acquity ™ Ultra Performance LC with a Waters UPLC BEH C18 (1.7µm 2.1 x 50mm) column; mobile phase A= ACN 40% + 0.1%FA, mobile phase B=ACN 100%+ 0.1%FA; strong needle wash= 70/20/10 MetOH/H_2O/IPA; weak needle wash ACN40% pump flow= 0.4mL/min; injection volume = 5µL; column temperature 50C; sample temperature= ; detection wave length= 318; gradient: t=0min 0% B; t=1min 0%B; t= 4 min 20%B; t=6min 60%B; t=9min=100%B t=10min 0%B; requilibration= 3min.

ESI- MS:
ESI-MS was connected to the same BEH C18 (1.7µm 2.1 x 50mm) column used for UPLC and the method 30_100B dual WL 220_318 was set in the ESI-MS configuration.

Lyophilizer
Buchi Lyovapor L-300.

Results

The purpose of this research was to explore the feasibility of synthesising a drug conjugate to deliver Venetoclax to kidney cells. The study also aimed to evaluate whether the conjugate can release the payload and analyse the toxicity and effectivity of the final conjugate in kidney cells in vitro.

The synthesis of such conjugate contains a biological component; LZM and a chemical component; the VNTO-Lx. Both components of the synthesis were worked out separately until the final stage of the synthesis, where both components were brought together to form the final product.

Due to solubility incompatibilities, the final stage of the synthesis failed, and a new alternative carrier must be introduced to propose a conjugate that could still meat the desired outcome. PAMAM dendrimer presents higher solubility profile in water and replaces the LZM in the synthesis. More details about how the synthesis changed to include PAMAM dendrimer as well as the outcomes of this research journey will be presented and discussed below.

Before diving into the synthesis itself, there was a characterization stage. Initially, it was necessary to determine an adequate wavelength to detect VNTO and to tune up a UPLC method that would allow for a proper identification of the initial compound as well as synthesis products.

After running a UV spectrometry the wavelengths 220nm and 318nm were selected. The solvent cut off was visible when measured at 220nm therefore, clearer chromatograms were obtained at 318nm and chromatograms at 220nm were kept as control. All chromatograms presented in this report are measured at 318nm.

Venetoclax is a very hydrophobic molecule, its solubility in water is just 0.000933 mg/ml. The changes expected to achieve through the synthesis might also modify the hydrophilicity of the final conjugate, making it more hydrophilic, therefore setting up a UPLC system that allows the visualization of products that would expectedly elute earlier was of great importance to start the project.

Initially the UPLC method suggested by Zigart and Casar (45) was implemented however, this method includes ammonium buffer that must be filtered every time to avoid damage in the UPLC column and can later interfere with mass spectrometers. Therefore, the method was simplified to an Acetonitrile (ACN) gradient. The method 40_100B dual WL 220_318 was initially implemented but VNTO would elute too early and finally changing

the gradient provided a better resolution. This method was named 30_100B dual WL 220_318 and it was used in all chromatograms.

The chromatogram of Figure 12 is the characterization of pure VNTO as molecularly depicted in Figure 2. The retention time of pure unreacted VNTO is 5.192min.

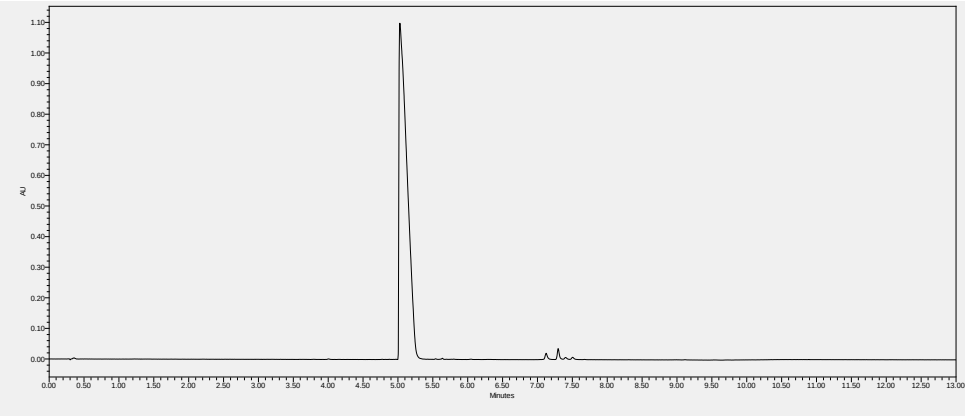

Figure 12. Pure VNTO Chromatogram. Method 30_100B dual WL 220_318. 318nm.

Once the UPLC method for detection of VNTO and possible synthesis products was set up, it was possible to continue with the next step in the synthesis: the activation of Dichloro(etylendiamine) Platinum II into the Lx.

The commercially available compound is a precursor of the linker which has 2 *cis*-oriented chloor groups, the activation of this compound is explained through the reaction of Figure 7. The Pt II centre has high affinity towards N and S donors even in biomolecules. In the presence of $AgNO_3$, a leaving Cl^- group is displaced by NO_3^- ions.

This active molecule will subsequently follow a complexation step by replacing the nitrate group for a more stable amine or sulphur donor, this complexation step occurs when the activated Lx is reacted with VNTO. This semifinal product has a positively charged Pt II center which provides it with a higher water solubility (46).

The characterization of the Lx poses a limitation to the UPLC method stablished. Other platinum compounds such as cisplatin which has a similar structure than VNTO absorbs with a maximum peak at 207nm (47), but not at 318 nm. Lx would likely absorb at a similar wavelength. Due to its hydrophilicity, it is expected that the Lx eludes early in the chromatogram, probably with the solvent front that is also not visible at 318nm.

Since the characterization of the LX compound was not possible with UPLC, ^{195}Pt Nuclear Magnetic Resonance (^{195}Pt-NMR) was then proposed. Despite several attempts to characterize the new compound using ^{195}Pt, the spectrums did not detect the molecule. This can be explained by the sample concentration required for the ^{195}Pt.

The isotope ^{195}Pt is naturally less abundant, in comparison the natural abundance of ^{1}H is >99.9% while ^{195}Pt abundance is around 33.8%. In addition, ^{195}Pt has a lower gyromagnetic ratio (γ^{195}Pt = 5.768×10^7 rad s^{-1} T^{-1}) compared to ^{1}H gyromagnetic ratio 2.675×10^8 rad s^{-1} T^{-1}, these arguments explain why ^{195}Pt NMR requires a higher

concentration (48,49). Disappointingly, the sample presented did not reach a concentration that could be measured using this technique.

In consequence, the characterization of the activated Lx was left to an indirect method; If the subsequent complexation reaction occurred (Figure 9) it would be visible using UPLC and the increase of the hydrophilicity of the new compound would result in an early eluding peak.

Figure 13 shows the chromatogram of the complexation product between VNTO and Lx.

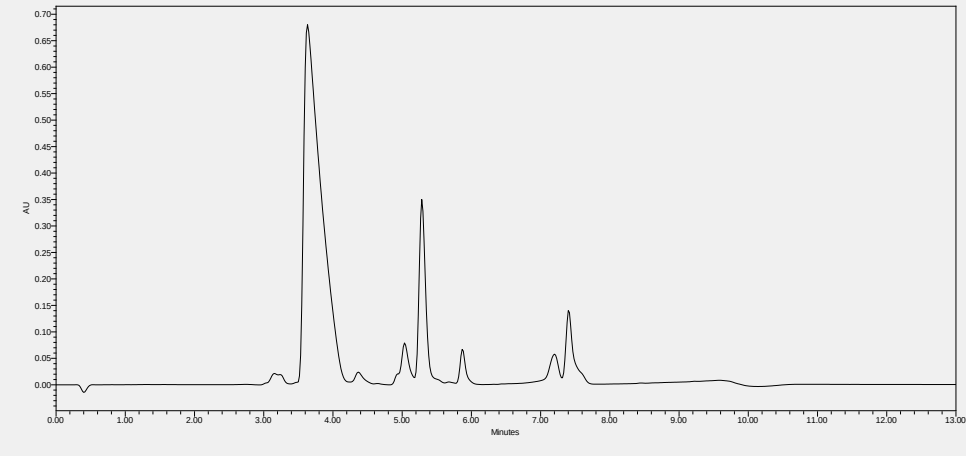

Figure 13. VNTO-Lx complexation chromatogram. Peaks: 3.641, 5.286 and 7.395 min. measured at 318nm.
*Unreacted VNTO: 5.192min

The chromatogram of Figure 13 shows that there are more species than the complexation product expected, the product is not 100% VNTO-Lx. Since the retention time of pure VNTO is 5.192min, the peak in this chromatogram at 5.286min corresponds to unreacted VNTO.

The widest peak eludes at an earlier time 3.641min, it is highly possible that this peak corresponds to the complexation product VNTO-Lx which is the product of interest for the consecutive reactions in this synthesis.

Later eluding peaks would represent more hydrophobic compounds, it could be possible that some fraction of the reagents has formed a new compound between one Lx linking to 2VNTO molecules. Platinum II usually adopts a square planar geometry (46) if 2 VNTO molecules are coordinated, the resulting molecule is also bigger which will contribute to a later eluding time.

A useful technique to purify the VNTO-Lx is HPLC prep, even degradation products of VNTO have been separated using HPLC-prep (45). During this research, we attempted HPLC prep with an acidic mobile phase as a purification method but the distance between the peaks was not enough to separate the fractions.

An analysis was conducted to determine through other ways to purify the molecule of interest. Various options were considered.

The addition of more Lx to react 100% of VNTO could cause a bigger residue of Lx. The excess of Lx is ready to react with any donors that could later come into the mixture. On the other hand, an excess of VNTO would difficult the quantification of the product at the end of the synthesis but it is unlikely to react in later stages.

It is important to consider what would be the impact of these residues in subsequent reactions in this synthesis route. An excess of VNTO does not react with met-LZM nor with any future reagents in the mixture whereas an excess of Lx could react with other donors. It hence seems feasible to use the crude reaction product without any further purification in the next conjugation step. The final conjugate must still be purified to remove unreacted VNTO and other species from the mixture and quantify the yield.

A strategy to avoid dealing with unreacted residues is to improve the reaction efficiency, to obtain a higher yield. A common technique to improve the reaction efficiency is carrying the reaction at a higher temperature. Figure 14 shows how the temperature affects the consumption of VNTO. Increasing the temperature decreases the time of reaction, but in some cases, temperature can also cause degradation products. The chromatogram pattern is the same for both temperatures, which indicates that the temperature does not influence the appearance of side reactions or degradation products. Pokar et al (50) found that degradation products of VNTO appear due to acidic hydrolysis and oxidative stress but temperature up to 80°C for 7 days does not degrade the molecule. Therefore, it was expected that the increase of temperature would only improve the yield and reduce the reaction time as can be seen on Figure 14.

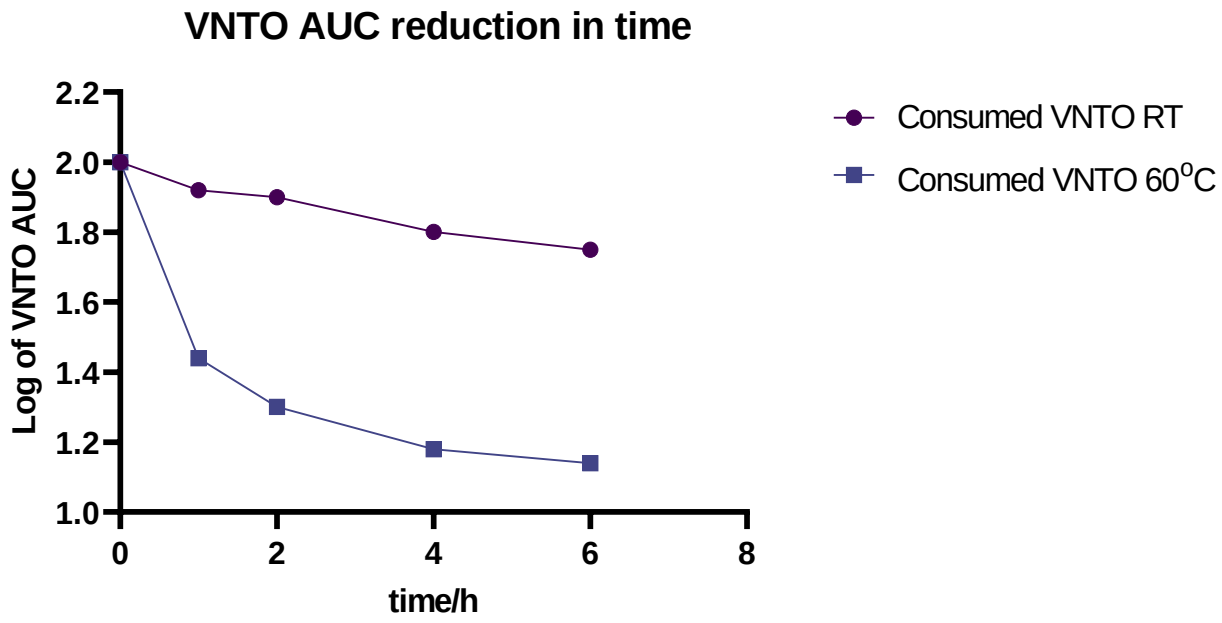

Figure 14. Temperature effect on the VNTO-Lx conjugation efficiency. Expressed as AUC

of VNTO peak (Retention time = 5.192min). Consumption rate constant at RT= 1.98. Consumption rate constant at 60°C = 1.72.
Note: RT means Room temperature.

One important research question is whether the linkage formed can release the payload in the presence of more reactive species in kidney cells. To mimic this release an experiment was carried out to displace VNTO from the Lx. This displacement is possible if another electron donor is added in excess, KSCN can donate electrons and bind to the Lx causing the displacement as shown on Figure 9.

Therefore, if the peak formed at 3.641min in the chromatogram on Figure 13 is actually VNTO-Pt the displacement would cause the disappearance of this peak and an increase of the signal at around 5.192min which corresponds to unreacted VNTO.

The displacement reaction was carried out using three different concentrations of KSCN 10 fold, 100 fold and 1000 fold excess. The displacement effect of these reactions are shown in Figure 15. After 24 hrs of reaction the displacement was complete when 100x fold and 1000x fold excess were present, while the reaction with 10xfold excess reached a 68% displacement after 24 hrs.

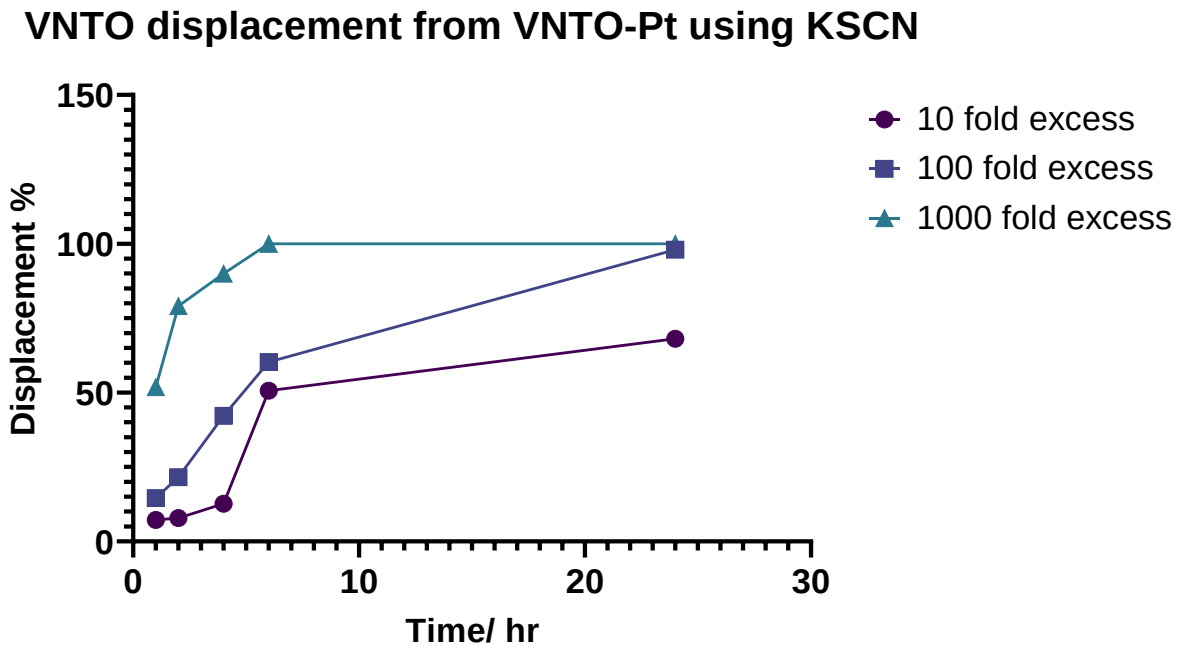

Figure 15. Speed of VNTO displacement from the VNTO-Pt using KSCN at different concentrations. Reactions carried out at 60° C. Calculated as the disappearance of the VNTO-PT peak. Initial VNTO-PT peak AUC= 16641361.

The importance of this displacement reaction goes beyond an indirect identification of the peak formed at 3.641min on Figure 13, the reversibility of this reaction is a good sign

for the application of the final product as a therapeutic agent since it is necessary to ensure the carrier can release the load. Just like the displacement competition demonstrated in this experiment, the release of drug conjugated systems using Lx technology is released in the lysosomes due to competitive displacement due to the interaction of Pt (II) with thiols (51).

In addition to the displacement reaction as a characterization test, the identity of the product of the reaction between VNTO and the Lx was later confirmed using Electrospray ionization mass spectrometry (ESI-MS). The results are presented in Figure 16.

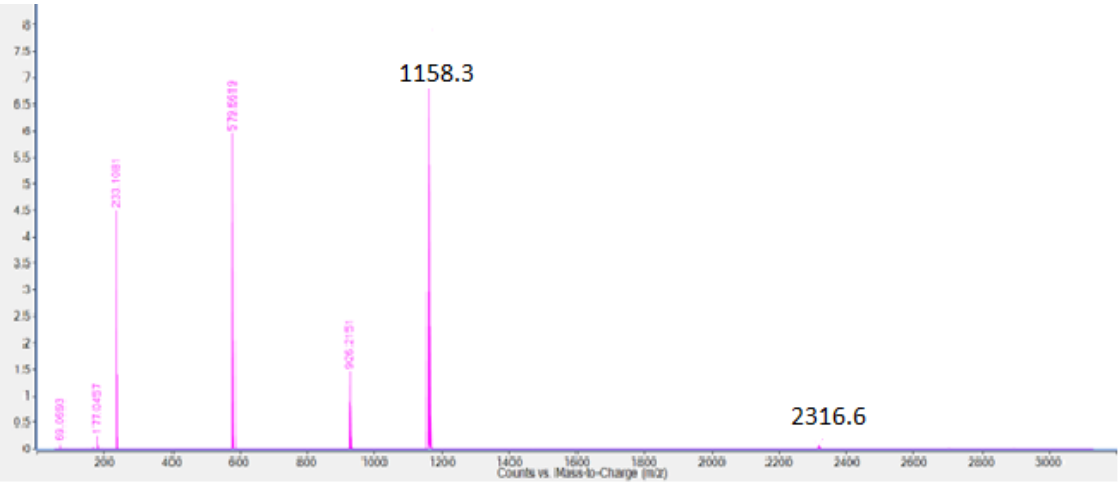

Figure 16. Electro Spray Ionization Mass Spectrometry of VNTO-Lx.
Peak size from left to right: 69.0693, 177.0457, 233.1081, 579.6619, 926.2151, 1158.3157, 2316.6245.

The deconvoluted peak eluding at 3.641min in chromatogram of Figure 13 has several charges and weights. The analysis of the peaks leads to supposition of possible fragments of VNTO or mass/charge possibilities.

The smaller the mass in the chromatogram the more difficult that it becomes to have certainty over their identity. For example, it could be supposed that the peak with molecular weight 69.0693g/mol could represent a fragmentation of the furan ring, furan ring has a molecular weight of 68.06g/mol, a derivate could account for the molar mass seen on the spectrometry, yet this is only a speculation.

Leaving speculation aside, ESI-MS gives certainty about the presence of VNTO-Lx since the peak at 1158.3 matches perfectly with the compound's monoisotopic mass when the molecule has only one charge. This peak is the confirmation that the desired complexation product between VNTO and Lx is formed.

There is an interesting relationship between the peaks 579.6619, 1158.3157 and 2316.6245. ESI-MS presents masses as a ratio of their charge (m/z). In case that the

compound of interest (1158.3157g/mol) has only one charge it will remain the same value on the spectrometry, but if this compound has a 2+ charge, it will be represented in the spectrometry as 1158.315/2 which corresponds to 579.6619 mass. Therefore, the mass 579.6619 is a fraction of VNTO-LX that was ionized with charged 2^{+}. See Table II.

On the other hand, if the mass of the peak of interest is doubled, and the +1 charge is maintained, then we obtain a mass of 2316.6245g/mol. It is likely that this fraction corresponds to an arrangement of 2VNTO-Lx molecules with a single charge, the molecules could be interacting with each other through their hydrophobic domains.

Table II. VNTO-Lx ESI-MS peak identity.

Molecular structure	Mass/charge (m/z)	Chromatogram peak
VNTO-Lx 2^{+}	1158.3/2	579.6
VNTO-Lx	1158.3/1	1158.3
2 (VNTO-Lx)$^{+}$	2(1158.3)/1	2316.6

The confirmation of VNTO-Lx in this mixture is a positive sign to move on with the other branch of the reaction. The modification of the LZM and its following conjugation with VNTO-Lx.

Unmodified LZM has 7 lysine (Lys) groups; the lysine at the N terminal of the protein and there are 6 additional side chain Lys (52). Commonly in drug development these terminals are modified to increase reactivity of the protein which in return improves efficiency and specificity of the reactions.

Other modifications to the Lys sites include the addition of cysteine, tyrosine, arginine or histidine. Some of these modifications might be more reactive for linking with the Lx (53). Nonetheless, BOC-Met-OSu is inexpensive, and Met has higher reactivity than Lys. The resulting modification was confirmed using ESI-MS. Figure 18 shows the resulting ESI-MS spectrometry.

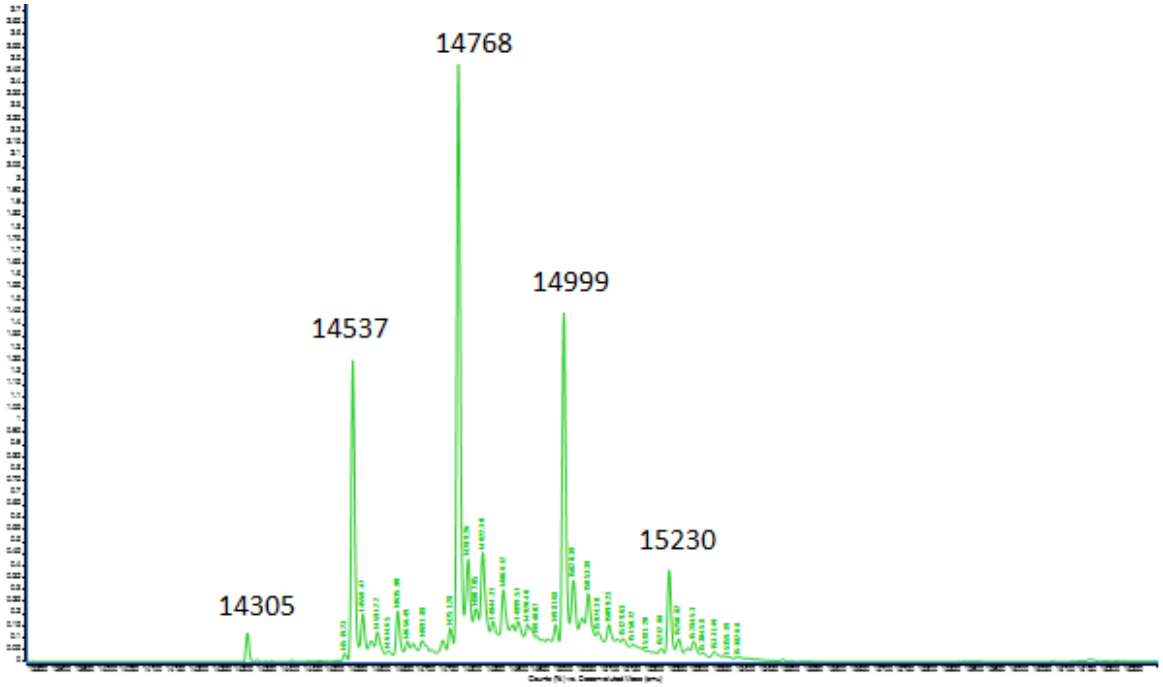

Figure 17. Methionine modification to LZM lysine terminal groups.

The initial mass of 14305 g/mol corresponds to the initial unmodified LZM. The main tall peaks after this initial mass, are separated from one another by a mass of 231g/mol which corresponds exactly to the monoisotopic mass of Met. Based on this analysis is possible to find in the sample LZM with up to 4 met groups.

The peak height of mass spectrometry cannot be seen as a proportion of the components in the mixture because some molecules might fragment during ionization, and the ionization efficiency may be different between components. Nonetheless, the components in this mixture would exhibit similar fragmentation and ionization patterns since the only change is the number of Methionine groups attached. Therefore, we induce that the 80th percentile of the mixture has between 1 and 4 methionine attached. The chemical interpretation is shown on Figure 19.

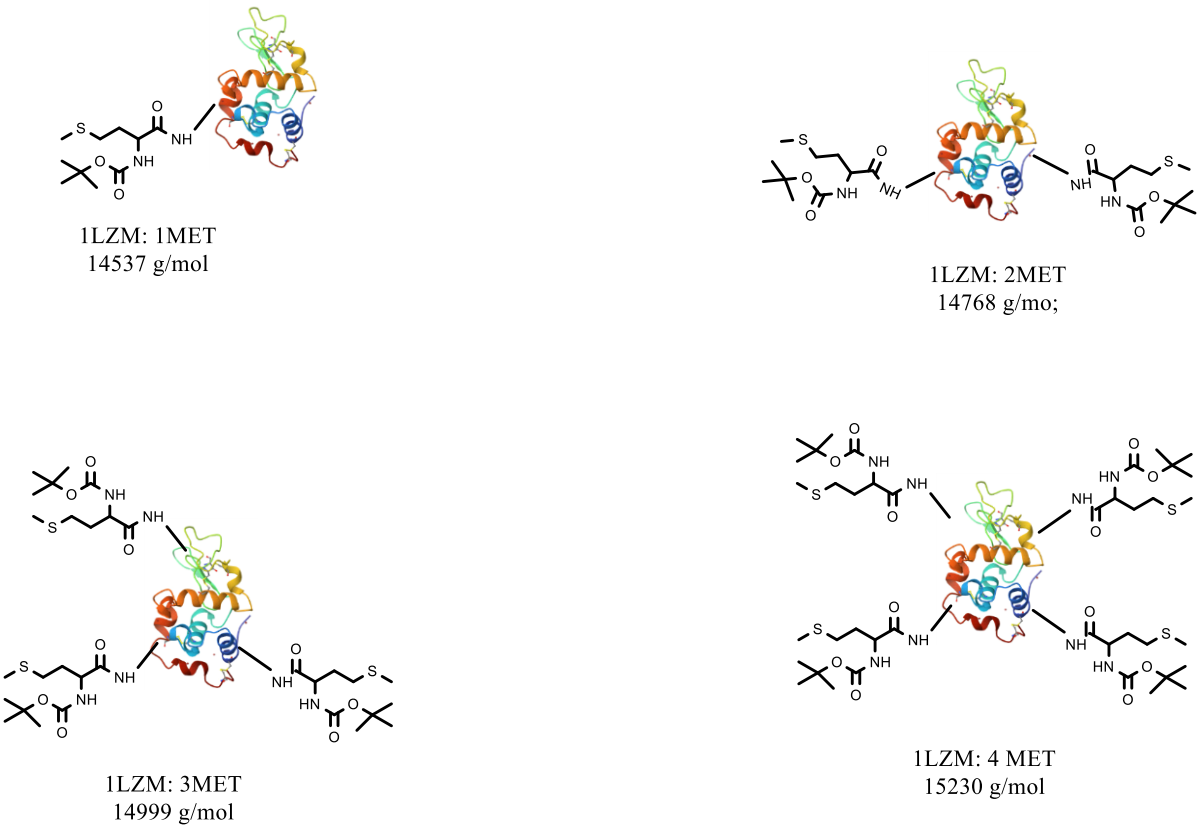

Figure 18. Weight and structural comparison of met modifications.

Interestingly, the smaller peaks between the jumps of 231g/mol in the spectrometry follow a similar pattern; the spacing between these peaks as well as the relative intensity follow a constant predictable pattern, these peaks could be attributed to isotopic patterns of the protein. Due to the natural abundance of ^{13}C, ^{15}N, ^{17}O, ^{18}O, ^{2}H and ^{34}S.

At this point of the research, both parts of the synthesis are completed. The identity of VNTO-LX and LZM-Met have been confirmed and both reactions were successful until this point. The intermediate products are ready for the final step of the synthesis: the coordination of VNTO-Lx with the met section of the LZM.

Based on the hydrophilicity differences between both components of this synthesis, it is expected that the final conjugation step would be challenging.

Literature points that efficient conjugation reactions using LZM-Met normally use a maximum of 20% of organic solvent (33–35), the final conjugation was initially attempted using 10% volume of DMF from the final mixture. The moment VNTO-Lx (in DMF) was added to the mixture of LZM-Met (in PBS), an homogeneous mixture was obtained, and when inserted in the dialysis cassette it remained homogeneously yellow until next day some precipitation was evident at naked eye. After dialysis, the mixture was filtered and it was evident that VNTO-Lx had precipitated or was in a suspension state (see Figure 19).

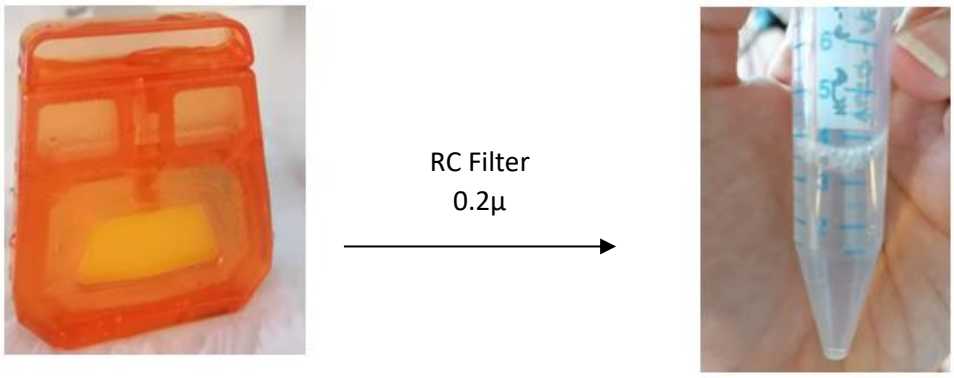

Figure 19. Reagent precipitation in the coordination of VNTO-Lx with LZM-Met.

To overcome this challenge PBS was replaced by water to remove salts and acidification of the reaction medium was proposed, nonetheless, the same results were obtained. The yellow precipitate was catched in the filter while LZM presence in the filtrate was confirmed by UV spectrometry.

Before moving forward with other attempts to overcome the solubility challenge, an experiment to determine the maximum proportion of DMF that LZM-Met could take without denaturalizing and the maximum of water VNTO-Lx could take before precipitating was conducted, this test would indicating the optimal solvent proportions for the final reaction.

In this experiment VNTO-Lx was dissolved in DMF and DMA and was exposed to different concentrations of water. Unfortunately, precipitation was visible at naked eye starting at 30% water whether VNTO was in DMF or DMA. VNTO-Lx concentration in solution was measured and the results are presented on Figure 20.

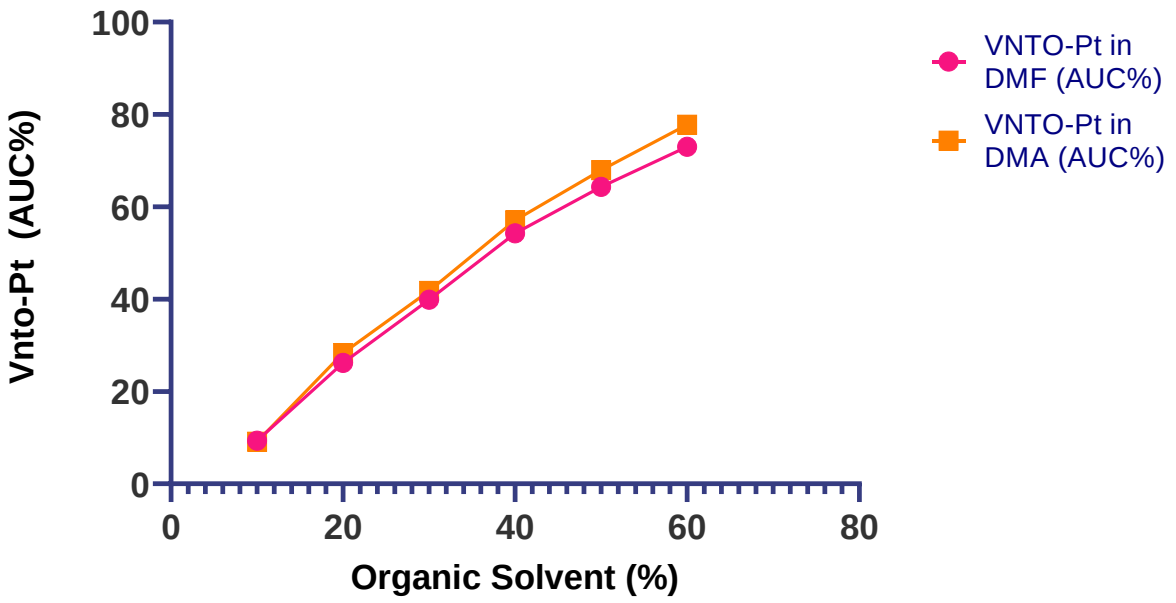

Figure 20. AUC percentage of VNTO dissolved at different organic solvent proportions.

VNTO structure contains benzene rings and alkyl groups that are in nature hydrophobic, and this characteristic also poses a challenge for the bioavailability of this drug alone. A strategy that could improve the solubility of VNTO is the addition of surfactants to the mixture (54). Some surfactants can also be added to the final formulation to avoid aggregation, the most used surfactants are polysorbate 20, polysorbate 80 and poloxamer 188 in concentrations that range between 0.001 to 0.1 w/v, nonetheless, the addition of surfactants could also destabilize the protein and could degrade the protein through the formation of reactive oxygen species (55,56). For the implementation of this strategy further experiments must be conducted to ensure the improvement of solubility whilst maintaining the protein integrity.

Another strategy to facilitate the reaction is carrying out a phase transfer catalysis. If VNTO-Lx is dissolved in a hydrophobic solvent, a 2 phase reaction could be attempted by increasing the surface area of the reaction.

Both strategies, the addition of surfactants as well as carrying the reaction in a phase transfer catalysis system were not implemented during this research but are worth taking into consideration as further steps to favour the feasibility of this reaction.

On the protein side, a similar solubility experiment was carried out to determine how much of organic solvent the protein would accept before precipitating.

LZM's natural colour is white, to facilitate the precipitation visualization, a staining with FICT was carried out. Precipitation as determined visually, occurred starting at 40%. Precipitation at 20% and 30% of organic solvent was confirmed with DLS (data not shown). The samples had agglomerates (Table III).

Table III. LZM visual precipitation at different organic solvent proportions.

Organic Solvent	10%	20%	30%	40%	50%	60%	70%	80%	90%
DMF	-	+/-	+/-	+	+	+	+	+	+
DMA	-	+/-	+/-	+	+	+	+	+	+

LZM precipitates with as little as 20% and VNTO precipitates with as little as 30% water. Sadly, these results confirm that solubility incompatibilities are too big to complete the final coordination step using the 2 components as they are. Further modifications of VNTO-Lx could improve the reaction result but due to limited time during this research, it is not possible to pursue that path.

The modifications of VNTO that could contribute to an increase of solubility are for example glycosylation and addition of polar functional groups such as in sulphonation (57,58). For this strategy is important to take into account the position of the addition since this could affect the therapeutic effect of VNTO.

Until this point the only research question or objective that has been answered is the release capability of the Lx to drop the load against competition. The main goal of developing a drug conjugate, its safety and effectivity must still be answered.

To answer the research objective, its necessary to reevaluate the synthesis components. The particle of clinical relevance is VNTO while LZM is only the carrier. Protein carriers have several advantages, especially their no toxicity and their easy elimination, nonetheless, choosing another protein carrier would imply the same solubility incompatibilities, therefore, a good workaround would be to use a non-protein delivery system. A good option would be to use a dendrimer.

Dendrimers are synthetic polymers that repeat units in the form of branches, their surface exposes charged functionalities that also determine their hydrophilicity level (59). The surface functionalities of the dendrimer can be linked with VNTO through the Lx.

Several dendrimers could be considered, for example polyamidoamine (PAMAM), polyglycerol dendrimers, (PEG) dendritic polyglycerol sulphate (dPGS).

PEG dendrimers are easily cleared through the kidney but they don't usually accumulate in the kidney, dPGS dendrimers contain sulphate that provide it with a very high negative charge which in return could cause nonspecific interactions in the blood stream and polyglycerol dendrimer might be too big to be effectively filtered through the kidney and they may need to be functionalized to achieve kidney delivery (60).

PAMAM dendrimers are among the most studied, research shows they can be used as delivery systems for renal therapies (61–63). Dolman et al found that PAMAM dendrimers can accumulate in proximal tubular cells of the kidney. From this family, ethylenediamine

core G3 PAMAM accumulates with the highest affinity (64). For this reason, an alternative synthesis was proposed using PAMAM dendrimer ethylenediamine core generation 3 as the carrier. From here on, this name will be shortened to Dendrimer.

Figure 21 shows the basic structure of the dendrimer. A third generation dendrimer has 32 amine reactive groups on the surface.

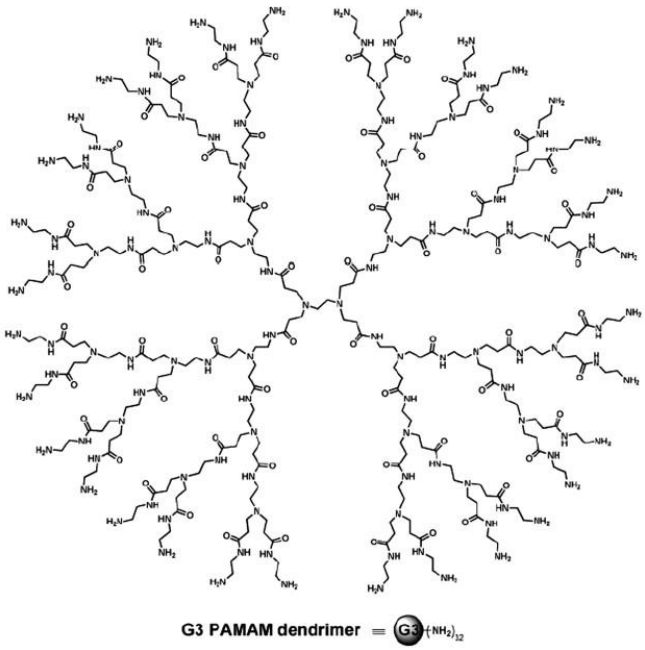

Figure 21. Diethylenediamine core PAMAM generation 3 (G3) dendrimer.
Figure taken from Charles et al (65)

Despite evidence shows that PAMAM G3 dendrimer is a good substitute for the LZM, it is important to compare the advantages and disadvantages of this change in the synthesis to account for possible new challenges. Table IV compares both delivery systems.

Table IV. LZM vs PAMAM dendrimer advantages and disadvantages.

Aspect	LZM	PAMAM Dendrimer
Reach kidney	LZM is filtered and reabsorbed in proximal tubule epithelial cells by the membrane receptor megalin in the kidneys (36)	Ehylenediamine core G3 PAMAM accumulates with high affinity in kidney proximal tubular cells (64)
Toxicity	Nontoxic and does not accumulate in the body	Hepatic or renal elimination, toxicity and accumulation depends on surface charges and size; cationic dendrimers and bigger size are usually more toxic (59,66)
Allergy capacity	Can induce a severe allergic reaction, including anaphylaxis (67)	Cationic dendrimers have higher risk to interact with immune system and cause inflammation
Structural compatibility in synthesis	Nitrogen in Methionine modification donate electrons for the coordination with Lx. Lx coordinates to a Sulphur atom	Has 32 reactive amine groups on its surface to bind with Lx. Lx coordinates to a Nitrogen

One of the main differences between these 2 systems lays on their safety profile. Both carriers have allergenic potential, but LZM does not accumulate while the dendrimer accumulation depends on its charge and size.

Another difference is the way both delivery systems coordinate with the Lx. LZM coordinates through a Sulphur which is less stable than the amine binding group of the dendrimer. This difference may have implications on the elimination/accumulation profile of the final products. It could be expected that the LZM conjugate would degrade in smaller fragments allowing easy exit to the cytosol. In contrast, the dendrimer conjugate has a stronger binding which could cause the Lx to remain bonded to the dendrimer inside of the lysosomal compartment, causing accumulation of dendrimer-Lx.

The elimination of dendrimers is usually hepatic or renal and their toxicity depends on the surface and the generation of the dendrimer. Despite Dolman et al (64) found no toxicity concerns for this dendrimer, it is important to keep in mind this possibility.

The use of a new carrier comes with new challenges that are important to evaluate in the final conjugate but first it's necessary to consider the feasibility of the synthesis itself. The synthesis method is presented next:

VNTO-Lx conjugation with Dendrimer.

The dendrimer PAMAM diethylenediamine core G3 (PAMAM) and VNTO-Lx were conjugated as follows: 5µmol of VNTO-Lx (5.79mg) were incubated with 5µmol of dendrimer (34.04mg) in 0.8ml of DMF. The reaction is calculated as 1mol VNTO-Lx per 1 mol dendrimer (with 32NH2 groups).

For calculation consider that the dendrimer is in 20%wt solution in methanol.

The reagents were incubated at room temperature with magnetic stirring in the dark during 4 hrs.

After 4 hrs of reaction, the peak that corresponds with VNTO-Lx of Figure 7 disappeared entirely and a new peak appeared right at the injection time as seen on the chromatogram of Figure 22.

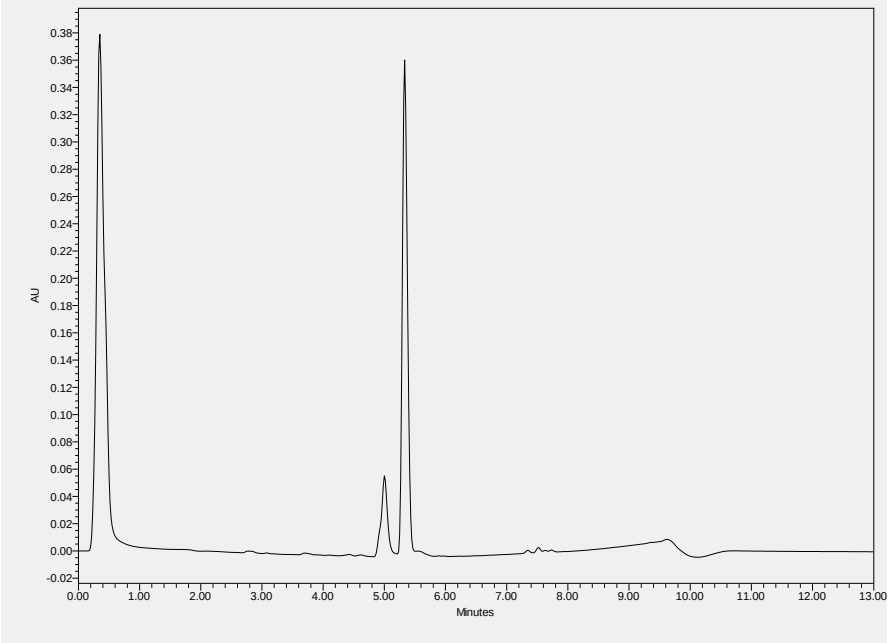

Figure 22. Chromatogram shows new product after VNTO-Lx-Dendrimer reaction
New peak RT: 0.340 min.

The dendrimer alone does not absorb in the UV range, therefore only when conjugated with a form of VNTO the dendrimer would be visible. It was expected that the conjugation with the dendrimer would increase the solubility of the final conjugate.

Figure 22 confirms the formation of a new entity that consumed VNTO-Lx completely. The new peak has an early retention time, the solubility of this new peak is so high that it comes right at the injection time.

As a reference test, staining the dendrimer (for example with FICT) would also give an idea of the elution time of dendrimer components.

The chromatogram of Figure 23 is a positive indication that the final conjugation between VNTO-Lx and the dendrimer was successful. Nonetheless, further characterization would confirm this conclusion and provide extra information as the conjugation ratio or any subproducts formed. Figure 24 is a representation of how the final conjugate would look like.

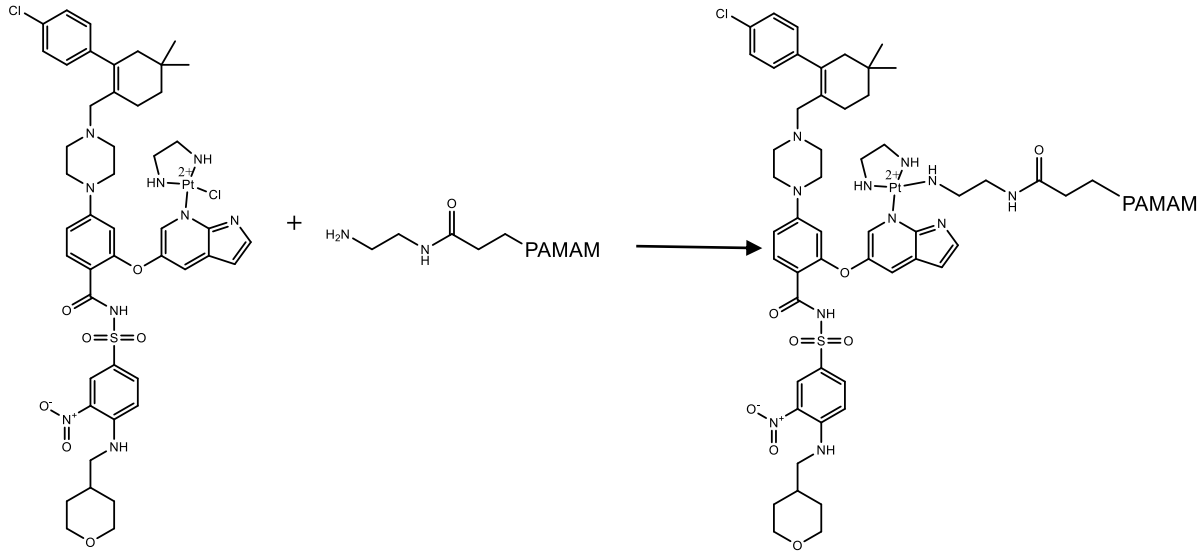

Figure 23. Final product of the conjugation of VNTO-LX-PAMAM.
MW: VNTO-Lx (1158.3g/mol), PAMAM dendrimer (6908.84 g/mol), VNTO-Lx-PAMAM (8030.74 g/mol).

Ideally a mass spec technique would be the most conclusive test. Initially, the sample was analysed with ESI-MS.

ESI-MS presents results as a ratio between the mass and the charge of the compound, and this poses a challenge for the identification of compounds that can have multiple charges such as the dendrimer, for this reason, the results were not conclusive and further characterization testing was performed.

A KSCN displacement reaction contributes to characterize the new product, if it reacts, the VNTO or VNTO-Lx peaks could be restored, then it's an indication of both: conjugation of the dendrimer with VNTO-Lx and the payload release capability of the newly formed conjugate.

The methodology for the dendrimer displacement test is explained below:

KSCN displacement of VNTO-Lx- Dendrimer

Three solutions of KSCN in DMF were prepared and incubated with the dendrimer conjugate at 60°C. KSCN was added in 10, 100 and 1000 fold molar excess to the dendrimer conjugate as shown on Table V . Samples were taken at 1,2,3 and 4 hours to monitor the progression of the reaction.

It is expected that the displacement could be partial or total. A partial displacement will increase the AUC of VNTO-Lx while a total displacement will increase the AUC of VNTO alone in the final chromatogram.

Table V. Excess of potassium thiocyanate used to displace VNTO from the dendrimer conjugate.

Conjugate : KSCN (mol : mol)	**Conjugate (mg)**	**KSCN (mg)**	**DMF (mL)**
1:10	2.3032mg	2.9154	1
1:100		29.1540	
1:1000		291.5400	

The graph of Figure 24 shows the progression of the displacement reaction through time using different concentrations of KSCN. At the end of the reaction the displacement of VNTO was close to an 80% completed.

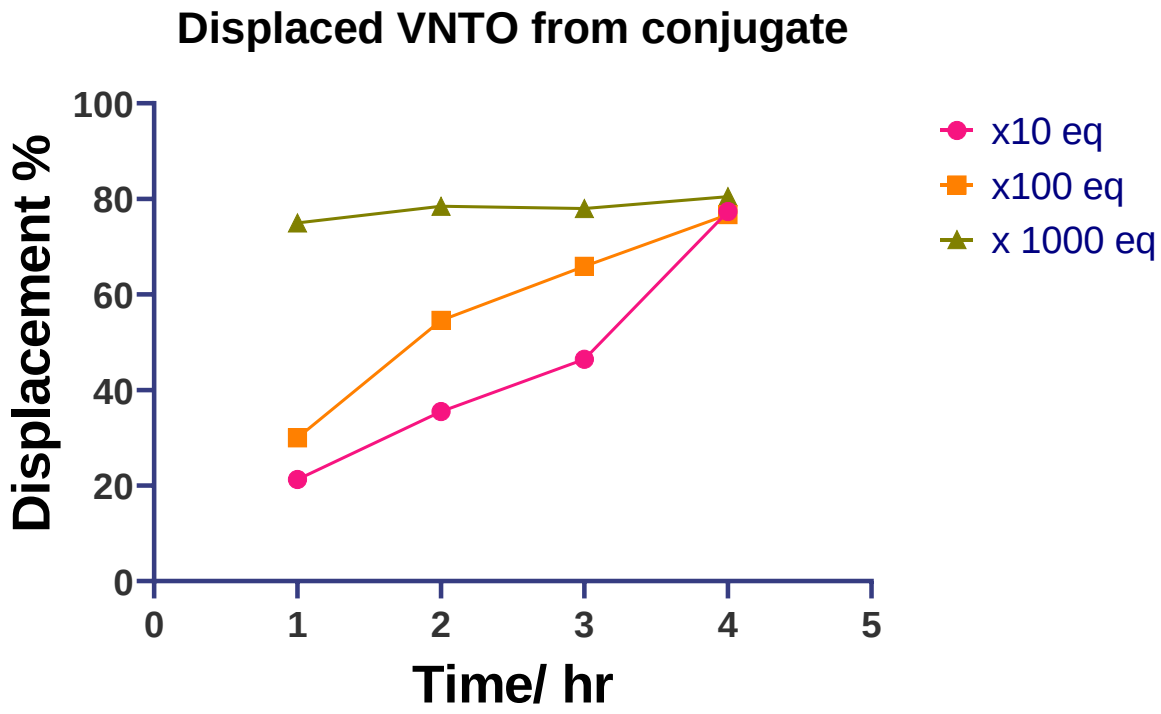

Figure 24. Displacement of VNTO from the VNTO-Lx-Dendrimer conjugate using KSCN in different excess concentrations at 60C.
Note: The reference peak used as 100% was the newly formed supposedly dendrimer conjugate.

Interestingly, despite the excess of KSCN only an 80% of dendrimer was displaced from VNTO. This suggest that other factors might be at play like the reaction reaching some equilibrium state where not all VNTO-Lx can be displaced.

Due to time limitations, the characterization of the new peak was only possible through the displacement reaction.

As a more conclusive characterization test, Maldi ToF was considered. On Maldi ToF, the conjugate mass is only presented with one charge. The effectivity of Maldi ToF in measuring the mass, depends on the selection of the proper matrix and the sample concentration.

Due to time limitations, Maldi ToF was the last test performed on the final conjugate and there was only one chance to perform the test. Unfortunately, in that occasion, the sample concentration was too high, which caused the sedimentation of the sample on the plate, in addition, only one matrix was tested (dihydroxybenzoic acid, 4-hydroxy-3-methoxycinnamic acid) and despite PAMAM dendrimer has been analysed with this matrix by Peterson et al (68), it might have been necessary to try other matrixes such as a-cyano-4-hydroxycinnamic acid, dithranol or DHB/fucose (68,69).

Another technique to assess the identity of the product is Size Exclusion Chromatography (SEC) or gel permeation chromatography, with this technique it is possible to obtain a

reference on the molecular weight as a function of the molecule's retention time once the column has been calibrated (70).

During this research SEC was not performed, the column to test the dendrimer had to be ordered and would not be available before the end of this project. Nonetheless, if Maldi ToF results are not conclusive, identification with SEC is highly recommended as a next step.

Once the identity of the final compound is confirmed, it is necessary to purify the final conjugate. The difference in solubilities between the residues of previous reactions (VNTO, VNTO-Lx, inactivated Pt II and Lx) and the conjugate at this point are quite drastic. This difference in solubilities can be used for purification. For small amounts of conjugate, UPLC-prep would probably show a more viable separation distance.

But for bigger amounts, the addition of water to the final mixture could cause the precipitation of VNTO, and the Pt compounds for a consequent filter and lyophilization. Membrane filtration, crystallization and chromatography could also be used at a bigger scale.

In addition to purification, the drug load in the conjugate could be increased. The dendrimer conjugate synthesis was carried out with a molecular equivalent proportion of 1 VNTO-Lx: 1 Dendrimer to avoid agglomeration, however, the dendrimer has 32 amine groups that could be used to load more VNTO-Lx. It would be worth to explore how much more load could be added while maintaining the integrity of the delivery system.

The safety and efficacy of the conjugate would be evaluated on human conditionally immortalized proximal tubular epithelial cells (ciPTEC) (71).

These cells would be tested on different concentrations from the order of μM to mM in series of 1:10 dilutions. If necessary, allometric scaling would have been applied to mimic more accurate physiological environment and obtain more information about the compound behaviour.

When testing in vitro, certain results are expected. The cellular death cause by VNTO only could be similar to that of the drug conjugate. This behaviour could be explained due to differences in steps like absorption, liberation and time of effect between the two drugs.

Unfortunately, due to time constraints, cell testing was not possible, but seen the possible paths to continue this research on, it is worth to test the safety and efficacy of the new compound in vitro

A possible barrier that could already be considered is the toxicity potential of the dendrimer conjugate. Some authors have found PAMAM dendrimers to be cytotoxic due to their surface cationic groups (72). To account for this possibility, the synthesis could be repeated using a biodegradable dendrimer.

Polyester biodegradable dendrimers are a good option for coordination linkage (See Figure 25). The carboxylic acid group on their surface can donate electrons to the Lx

making the reaction feasible in theory. While this modification can overcome any toxicity limitations, the reaction feasibility must be evaluated in the practice, just as the toxicity and effectivity of the final conjugate.

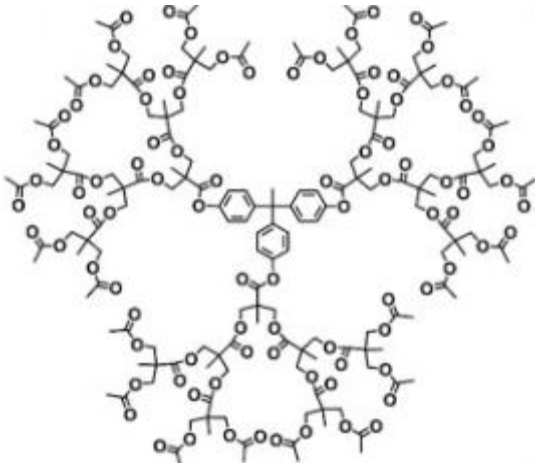

Figure 25. First reported polyester biodegradable dendrimer based on bis-HMPA.
Taken from Huang and Wu (72).

The cavities formed between the branches of polymer are suitable for the encapsulation of hydrophobic compounds. This is another path that can be studied to deliver VNTO in kidney cells. Other researches have also use dendrimer cavities for the encapsulation of drugs (73,74). This strategy could result in a conjugate with a controlled release of the load, it will improve the solubility of the drug, and it can also be targeted to the kidneys in case the unmodified dendrimer does not accumulate in the kidney.

The practical scope of this research was limited by the time it took to deal with the solubility differences between LZM and VNTO-Lx. Nevertheless, evidence of the formed conjugate using the dendrimer makes this line of research a promising answer to kidney diseases. In addition, the recommendations and synthesis alternatives herein presented guide on further options to improve the resulting conjugate and finally develop a senolytic therapeutic agent to regenerate kidney functionality.

Conclusion

This research studied the feasibility of synthesizing a VNTO conjugate targeted to kidney cells to cause senolysis and restauration of kidney function. The study also aimed to test the reversibility, toxicity and effectivity of the resulting compound.

The synthesis path described and the tests performed conclude the formation of a drug conjugate that binds VNTO to PAMAM dendrimer through Lx coordination chemistry. Evidence provided also confirms the linkage is reversible which suggests the conjugate can release the load.

Due to time limitations, the safety and efficacy of the obtained conjugate was not assessed in vitro, which leads to the conclusion that the absolute objective of this research was partially achieved.

This manuscript leads to future steps to purify and test the safety and efficacy of resulting compound as senolytic agent. It also recommends repeating the synthesis with a degradable dendrimer to account for possible accumulation and toxicity.

Extra conclusions can also be drawn from this research. Solubility differences between LZM and VNTO are so drastic that the final conjugation is unfeasible. Acidification and salts do not influence the solubility of VNTO-Lx significantly enough to make this reaction feasible. LZM precipitation begins at a 20% of organic solvent. Using these two components would only be possible if prior modifications are performed to improve the solubility profile of VNTO, but this could also affect its therapeutic effect.

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Use of Generative AI

Generative AI was not used for the elaboration of this report.

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