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ANNEXIN A5–METHOTREXATE: A NOVEL TARGETED THERAPEUTIC AGAINST BREAST CANCER AND LEUKEMIA

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ANNEXIN A5–METHOTREXATE: A NOVEL TARGETED THERAPEUTIC AGAINST BREAST CANCER AND LEUKEMIA

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ABSTRACT

One of the most challenging issues of chemotherapy in cancer treatments relies on specifically targeting and carrying the drug to the tumor cells. While tumor ablation surgery can be efficient, metastatic cancers are more challenging to treat because of the spread distribution of tumor cells in the body. Methotrexate is an FDA approved antimetabolite drug for the treatment of breast cancer and acute lymphoblastic leukemia. However, despite several conjugation attempts to optimize its delivery to cancer cells, methotrexate still does not offer a reliable enough therapeutic effect in the clinic.

We have investigated the conjugation of methotrexate to annexin A5, a human protein that specifically binds to the cancer cell marker phosphatidylserine. Once conjugated to annexin A5, methotrexate's cytostatic effect would be specifically delivered to cancer cells without causing off-target damage to healthy cells. This novel drug conjugate has been designed as a response to the currently urgent clinical needs for a targeted and specific chemotherapeutic treatment for triple negative breast cancer and lymphoblastic leukemia.

The successful conjugation of methotrexate to annexin A5 was confirmed by measuring the final concentration of annexin A5 and methotrexate in the conjugate. The increase in size of the conjugate was confirmed by SDS-PAGE. Cell viability assays of free and conjugated methotrexate were conducted on both tumor and non-tumor cells. The conjugate induced a toxicity that was statistically equivalent to the one delivered by the free drug on the triple negative breast cancer 4T1 and EMT6 cell lines, and on the lymphoblastic leukemia P388 cell line. However, while free methotrexate induces a cell mortality in healthy endothelial HUVEC and HAAE-1 cells, the conjugate does not cause any significant decrease of the cell viability, confirming its specificity towards tumor cells.

INTRODUCTION

Cancer disease

Cancer can be defined as a disease based on a group of anarchic cells that circumvent the physiological and homeostatic rules of the organism. While every healthy cell is subject to a permanent control and regulatory signaling, tumor cells have developed a certain independence that allow them to proliferate at an uncontrolled rate. Cancer cells are characterized by general hallmarks related to their cellular behavior: sustaining a proliferative signal, escaping growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis[1–4]. By sustaining their proliferative signals and evading the growth suppressors, they enable themselves to keep a high proliferative rate, provide themselves with the necessary growth factors and evade growth suppressors checkpoint of the cell cycle through the altered expression of oncogenes[5]. To further promote its growth, the tumor will considerably increase its vasculature by diffusing several angiogenic growth factors such as the vascular endothelial growth factor (VEGF) and the fibroblast growth factor (FGF). This angiogenesis induction involves the proliferation and migration of endothelial cells to the tumor site to supplement the cancer cells with nutrients and oxygen[6]. Through a considerable flexibility of their adhesive properties to other cells and to the extracellular matrix (ECM) that allows them to migrate from their initial site, cancer cells are transported in the bloodstream and promote secondary tumoral sites that can potentially lead to metastasis[7–10] (*Figure 1*).

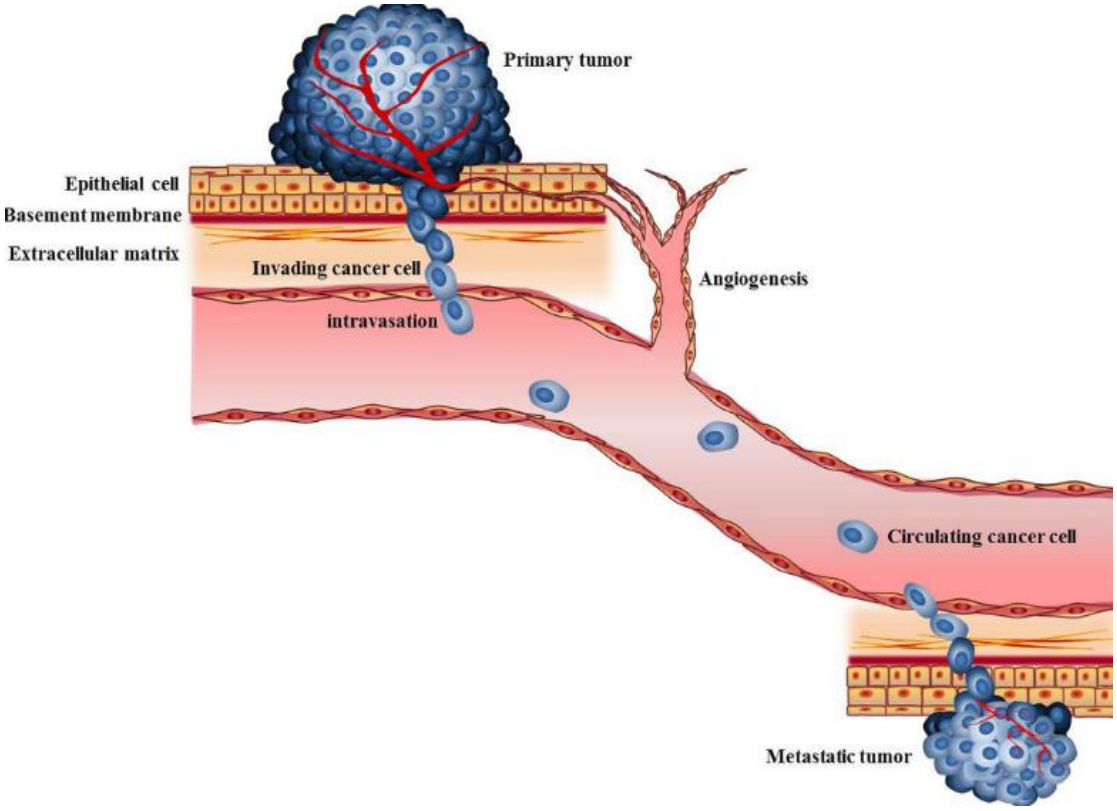

Fig. 1 Tumor evolution to metastasis stage. The primary tumor cells in the epithelial tissue proliferate, invade the extracellular matrix, cross the basal membrane, and penetrate the blood vessels. Once in the bloodstream, the cells are disseminated throughout the body and some cells exit the circulation by extravasation in secondary tumor sites, that proliferate into metastasis[10].

Breast cancer

Breast cancer is a type of carcinoma, a tumorigenic epithelial tissue. It is the 3rd leading cause of death worldwide for females between 50-59 years old[11]. It can be classified into several subtypes according to its hormonal receptor's expression (estrogen, progesterone and HER2 receptors) on the plasma membranes of the tumor cells. Breast cancers that do express these receptors are eligible for endocrinal therapy that present a certain specificity towards tumor cells. A subtype of breast cancer does not present any of these receptors which renders its treatment particularly challenging. Triple negative breast cancer (TNBC) accounts for 15% of breast malignancies. If an early stage of the disease can be

well addressed by surgery, metastatic and advanced-metastatic tumors are more complex to treat. Because of the absence of specific treatment guidelines such as endocrine therapy, the TNBC subtype is treated with standard therapies often associated with a high rate of local and systemic relapse. The undesirable side-effects observed in chemotherapies represent a clinical limit and impair the delivery of the anticancerogenic effect of the drugs[12].

Leukemia and lymphoma

Leukemia and lymphoma are the two main types of hematological malignancies. As a liquid tumor, cancer cells' distribution is systemic, and the metastasis occurrence risk is high. Lymphoma is exclusively based on lymphoid cell lines, the T and B lymphocytes, while leukemia can be based on both lymphoid and myeloid cell lines. Leukemia is classified into acute or chronic leukemia according to the maturity of the tumorigenic bone marrow cells and is located in the blood circulation. Lymphoma on the other hand, affects the lymphatic system through tumorigenic lymphocytes B and T and can be divided into Hodgkin (malignant B cells based only) and non-Hodgkin (both B and T malignant cells) lymphomas[13–18].

Methotrexate as an anticancer therapeutic

Methotrexate is a chemotherapeutic that has been used in the clinic for over 50 years. It is a folate derivative analog that interferes with the *de novo* synthesis of nucleotides and amino acids. Methotrexate is prescribed for the treatment of inflammatory diseases such as arthritis, psoriasis, Crohn's disease but is also an FDA approved chemotherapeutic agent for a great number of malignancies such as breast carcinoma, acute lymphoblastic

leukemia, non-Hodgkin lymphoma, osteosarcoma, head and neck cancer, and lung cancer[19].

With a very similar chemical structure to folic acid, methotrexate consists of a pteridine-diamine core linked to a glutamic acid moiety that contains two highly ionizable carboxylic acids, circled in red in *Figure 2*. However, its solubility in aqueous solvent is pH-dependent and requires neutral or basic pH values[20]. As a free drug, methotrexate is internalized into cells through two types of receptors: RFC, the reduced folate carrier, to which it has the highest affinity and the folate receptor FOLR1[21–24]. When methotrexate is internalized in the cell, it is converted to methotrexate polyglutamate by the enzyme folylpolyglutamate synthetase (FGPS) that adds glutamate molecules at the γ-carboxyl moiety of methotrexate (*Figure 3*). If the mono-glutamate form of MTX has a high affinity with a number of folate export processes, the polyglutamylation of methotrexate prevents its efflux and allows for an accumulation of the antifolate drug in the cell and a sustained inhibition of its targeted enzymes for long intervals[24]. The enzyme dihydrofolate reductase (DHFR) is responsible for the reduction of folic acid (FA) to tetrahydro folic acid (THF), which is the active component necessary for the biosynthesis of thymidylate and purine nucleotides. By competitively inhibiting the dihydrofolate reductase enzyme, methotrexate leads to a deficiency in the cellular pool of tetrahydrofolate and prevents the synthesis of dAMP and dGMP nucleotides. As shown in *Figure 3*, methotrexate also

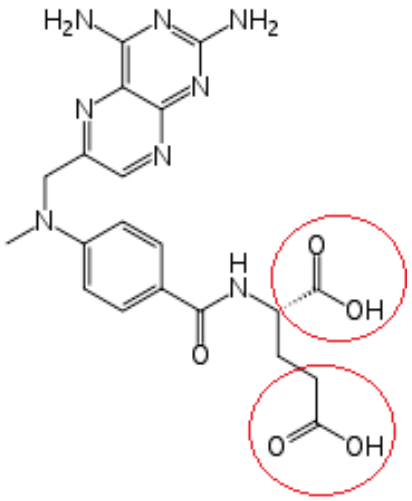

Fig. 2 Chemical structure of methotrexate. The two targeted carboxylic functions for conjugation to annexin A5 are circled in red.

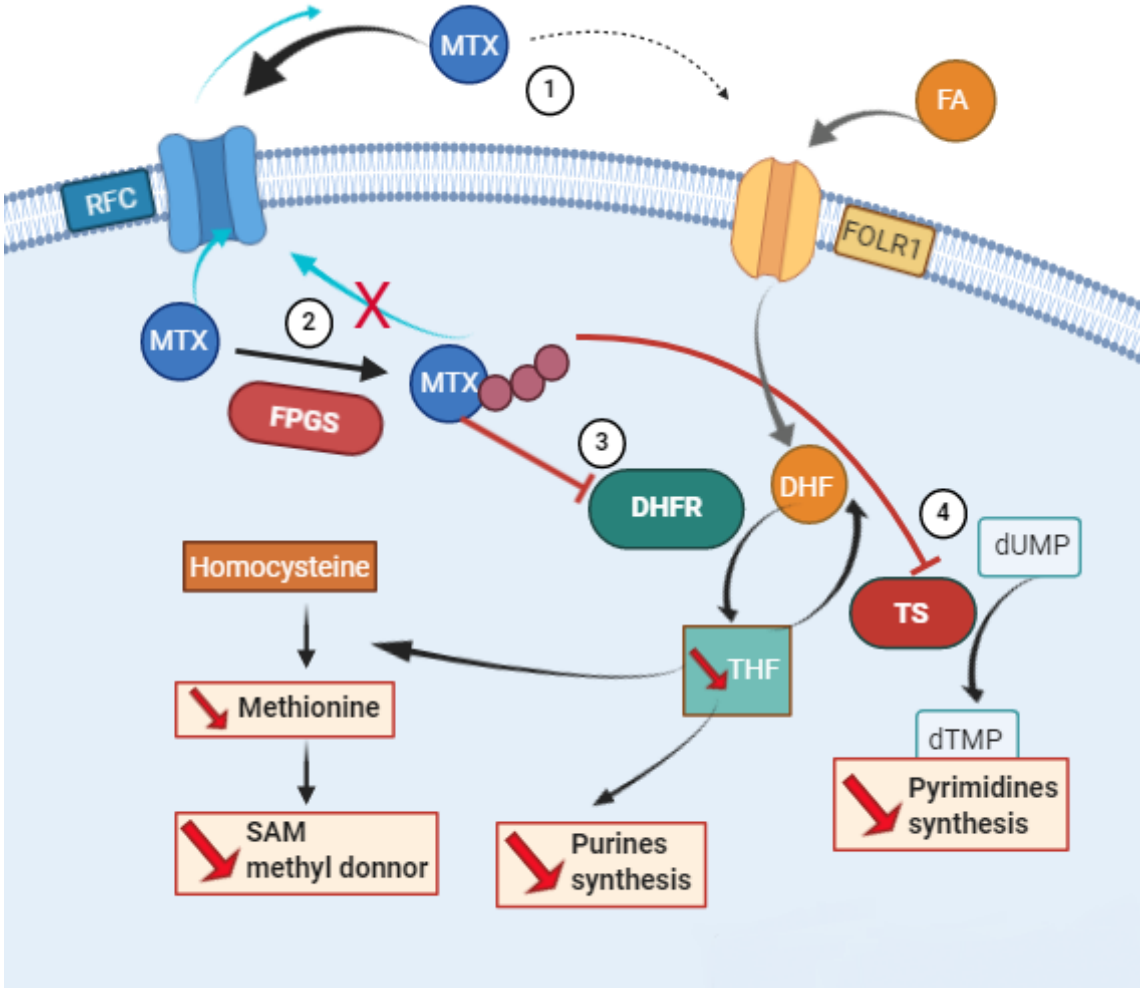

Fig. 3 Mechanism of action of methotrexate. (1) Methotrexate cellular uptake is mediated by the reduced folate carrier (RFC) to which it has the highest affinity and the folate receptor 1 (FOLR1) to which folic acid (FA) has the highest affinity; (2) Folylpolyglutamate transferase (FGPS) incorporates glutamates on MTX and promotes its accumulation in the cell; (3) MTX inhibits dihydrofolate reductase activity (DHFR) and decreases the pool of tetrahydrofolate (THF) leading to a decrease of purine synthesis and of the repletion of methionine and of the major methyl donor S-adenosylmethionine (SAM); (4) MTX inhibits the thymidylate synthase enzyme responsible for the production of thymidylate (dTMP), an essential component of DNA. The figure was drawn using the software © *BioRender 2020.*

inhibits thymidylate synthase (TS) that is responsible for thymidine synthesis, one of the two necessary pyrimidines for DNA replication and repair. Both decrease of THF and inhibition of TS lead to a depletion of thymidylate pool. Moreover, tetrahydrofolate is an

essential component for methionine synthesis through the provision of its methyl group to homocysteine. By depleting the available amount of methionine, methotrexate impairs the repletion of S-adenosylmethionine which is the universal methyl donor in the cell, paramount in nucleic acids expression regulation by methylation. Preventing the repletion of the available pool of methionine also limits the overall protein synthesis of cancer cells, as methionine is the essential amino acid that initiates the protein synthesis. Therefore, the antimetabolite methotrexate interferes with mitotic cell division, DNA repair and RNA and protein synthesis by depriving the cell of its essential nucleotides and amino acids biosynthetic pathways[25,26].

The cytotoxic effects of methotrexate occur during the S phase of the cell cycle due to the higher dependence of the cells on purine and pyrimidine *de novo* synthesis making the drug mostly active on rapidly multiplying cells. Indeed, malignant tissues display a higher proliferation rate and therefore an increased sensitivity to methotrexate action than normal tissues. This difference of sensitivity has been for a long time used to consider the drug delivery as specific. However, it is known today that free methotrexate administrations do not show a satisfactory specificity towards cancer cells and cause deleterious side-effects in treated patients. Indeed, tissues and cells that physiologically go through a high proliferative rate present a considerable sensitivity to methotrexate: bone marrow cell lines, fetal cells, buccal cells, intestinal mucosa or bladder urinary cells[27]. These are consistent with the most related side-effects of methotrexate: bone marrow suppression, oral and gastrointestinal ulcers, cutaneous toxicity, but also include acute renal failure, neurotoxicity and pulmonary inflammation[28].

However, the lack of specific addressing of MTX limits its therapeutic application in the clinic[29–31]. Several conjugations of MTX have been tested and showed a higher cytotoxic

effect when associated with nanoparticles, lysine polymer, albumin, fibrinogen, or lipo-amino acids. But none of them showed a sufficiently reliable targeted delivery of the drug and limited off-targets towards healthy cells[27,28,32–56].

It is known that when administered freely, less than 1% of a given drug will reach the tumor site and deliver an anticancer effect; the rest of the fraction is disseminated in the organism's tissues[57,58]. Methotrexate is easily metabolized by the organism and its fast blood clearance limits the tumor exposure to the drug. By combining a chemotherapeutic drug to a targeting carrier agent, its distribution to the tumor site can be optimized[59], which has been observed in albumin conjugates and nanoparticle drug therapeutics[35,40–43,60]. Targeted therapies rely on the specific delivery of the chemotherapeutic drug to the tumor site, preserving the healthy cells from undesired off-target effects and allowing for a lower efficient dose of the drug. Conjugating MTX with a carrier agent specific to tumor cells would ensure an optimal and targeted delivery of the molecule to cancer cells specifically.

Annexin A5 as a targeting drug carrier

Phosphatidylserine

Phosphatidylserine (PS) is a cancer cell targeting biomarker. It is an essential phospholipid ubiquitously present in human cells, expressed in the inner leaflet of the plasma membrane. Phosphatidylserine externalization is regulated by the activity of a calcium-dependent flippase. This enzyme ensures the rotation of PS towards the cytoplasmic side while floppase catalyzes the opposite reaction. The higher the intracellular calcium concentration is, the lower the flippase activity gets, promoting the externalization of PS on the outer leaflet of the plasma membrane[61]. PS is externalized in apoptotic cells and in a large majority of malignancies. Phosphatidylserine

externalization is a conserved evolutionary immunosuppressive signal that prevents local and systemic inflammation. Apoptotic cells externalize PS, circumvent an immune activation of lymphocytes and send an "engulf me" signal to macrophages that will bind to the ligand PS and proceed to the physiological efferocytosis[62–68]. Tumor cells utilize this immunosuppressive signal by mimicking an apoptotic signal through PS externalization and by expressing surface proteins such as CD47 that discourage the macrophages from engulfing them[69,70]. This pacification of phagocytic cells combined with the suppression of active immune cells recognition (T-cells and B-cells) is the hallmark of the established immunosuppression of tumor cells.

PS is described as an "underestimated Achille's heel of cancer" because of the high dependence of tumor maintenance in the organism and because of its wide expression in cancer pathology. Indeed, PS has been found to be externalized not only in primary cancer lesions, but also in metastasis, tumor vasculature, and primary cancer cell lines[68,71–73].

Annexin A5

Annexin A5 (ANXA5) is a member of the membrane binding proteins involved in different regulatory functions as a powerful anticoagulant and a cell surface coating agent. ANXA5 is an endogenous human protein of around 36 kDa that can bind to PS and therefore constitutes a cancer targeted carrier agent. The annexin A5-FITC fluorescent conjugate has been commonly used for its applications in flow cytometry and fluorescence microscopy for apoptotic cell death assay[74–79]. ANXA5 binding to PS is Ca^{2+} dependent[80,81] and is followed by an internalization in the cell, described as a novel macropinocytosis pathway that does not involve any interaction with the actin cytoskeleton. ANXA5 is made of a core of α-helices with a Ca^{2+} binding domain, a PS

binding domain, and an ANXA5 binding domain allowing for the formation of trimers. Once bound to PS, ANXA5 forms a trimer that binds to another ANXA5 trimer leading

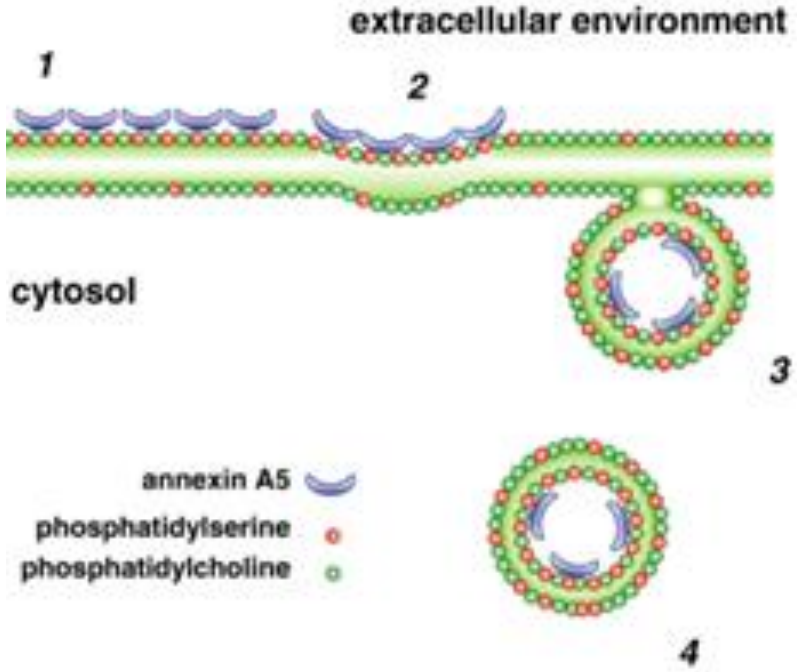

Fig. 4 Binding and internalization of annexin A5. Annexin A5 binds to phosphatidylserine (orange) and is internalized in cancer cells[82].

to a hexamer that forms a two-dimensional lattice. As shown in *Figure 4*, ANXA5 ultimately invaginates the plasma membrane followed by the intracellular trafficking of the endocytic vesicle[71,72,82–88].

Interest of conjugating methotrexate to annexin A5

The pharmacokinetic properties of methotrexate (fast metabolization and short blood clearance) greatly limit the tumor exposure time and the delivered antineoplastic effect. This leads to an increase of applied doses in the clinic that usually cause acute side effects and chemoresistance mechanisms. By conjugating MTX to ANXA5, the clearance time of MTX in blood will be extended, besides acquiring the cancer specific targeting of

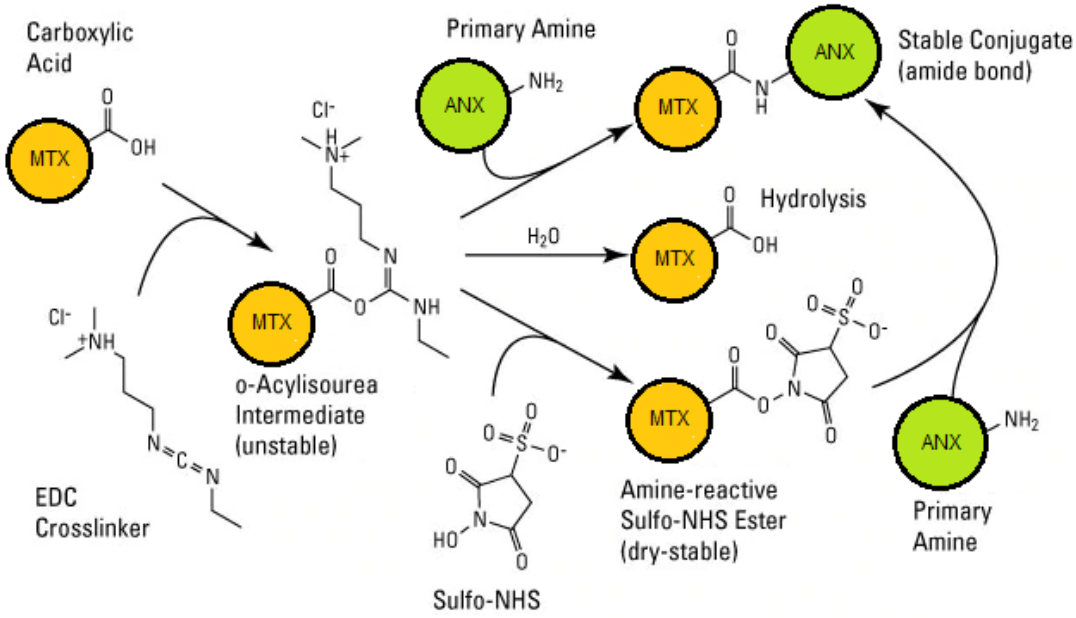

Fig. 5 Conjugation chemistry of methotrexate (MTX) and annexin A5 (ANXA5) using the coupling agent EDC and NHS. This conjugation reaction leads to a stable amide bonded conjugate. Source: https://www.thermofisher.com/us/en/home/life-science/protein-biology/proteinbiology-learning-center/protein-biology-resource-library/pierce-proteinmethods/carbodiimide-crosslinker-chemistry.html

annexin A5. The conjugation will rely on EDC-NHS conjugation protocol that allows for a zero-length carboxyl to amine cross-linking between the carboxylic acids of methotrexate and the primary amines of annexin A5 (*Figure 5*). This method of conjugation has already been successfully utilized in our lab for the development of several novel drug conjugates[89–91]. The methotrexate molecules will be conjugated to annexin A5 with amide bonds, providing a high chemical stability to the conjugate and preventing an undesired early cleavage of the drug. The annexin A5 triggered endocytosis has been reported to involve the lysosome. The acidic pH of the lysosome and the powerful enzymatic load contained inside are expected to cleave the conjugate's amide bond and release MTX in the cytosol[92] *(Figure 6).*

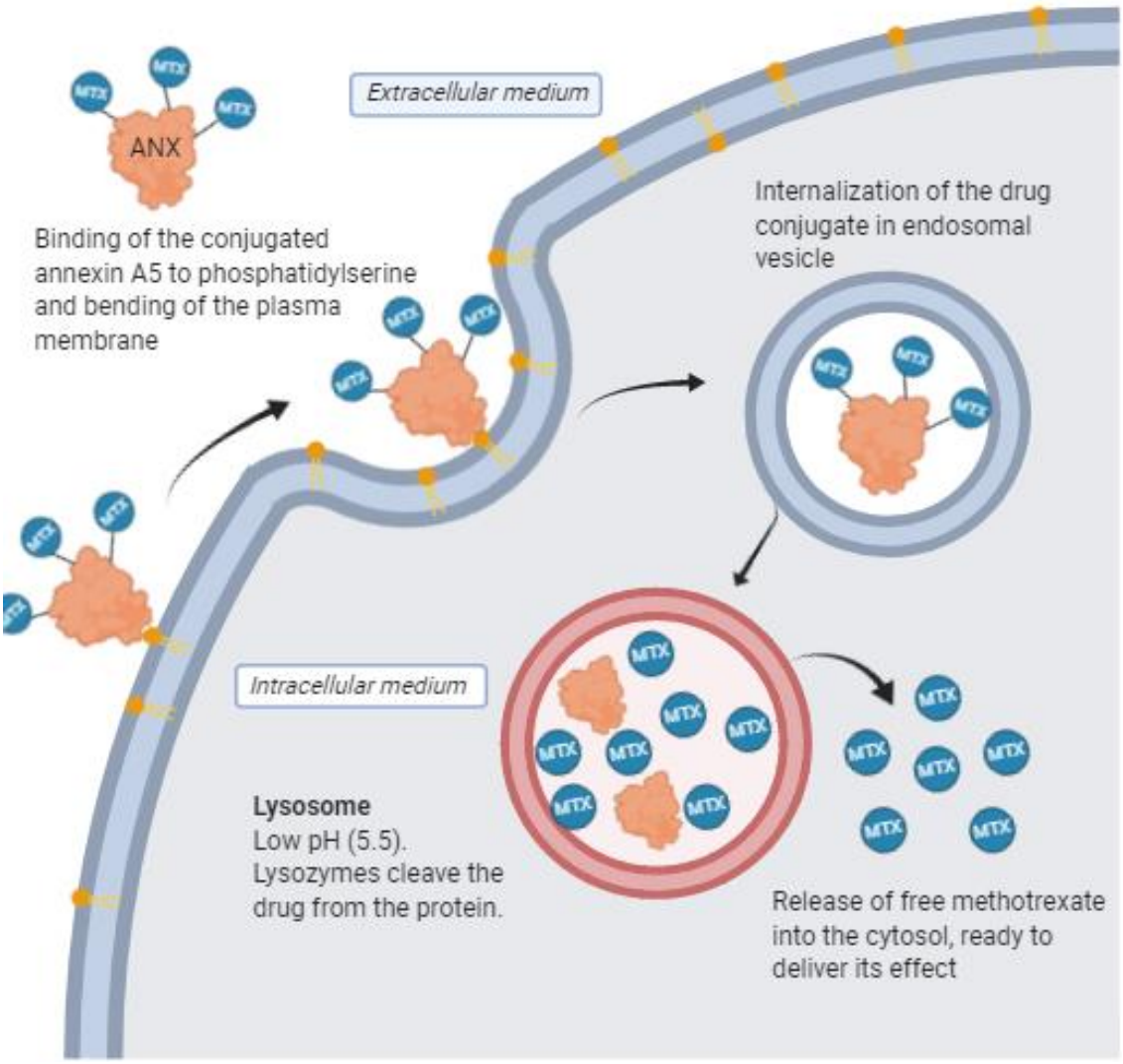

Fig. 6 Binding and internalization of ANXA5-MTX conjugate in cancer cells. The drug conjugate binds to externalized phosphatidylserine (yellow) of cancer cells, internalizes and releases active methotrexate in the cytosol. The figure was drawn using the software © *BioRender 2020.*

Scope of thesis

Hypothesis

We base our work on the combination of two biological properties: the ability of annexin A5 to bind to phosphatidylserine and to be internalized in the cell and the cytostatic function of methotrexate. Phosphatidylserine being considered as a specific cancer cell marker, our hypothesis relies on the preservation of the properties of the two molecules once conjugated. The elaboration of a successful conjugate would allow an effective binding and endocytosis in the cancer cells and a successful delivery of methotrexate antineoplastic effect, with negligible toxicity in healthy cells.

First objective: Elaboration of a stable annexin A5-methotrexate conjugate

The conjugation chemistry using EDC/NHS coupling is a reliable method to conjugate two proteins together and should allow for a good conservation of ANXA5 and MTX properties. The conjugates bands on SDS-PAGE are expected to be higher than the one of free ANXA5 to confirm the successful conjugation. The final conjugate solution must show good solubility and viability of the novel targeted therapeutic.

Second objective: In vitro assays of the conjugate on tumor and non-tumor cells

The assessment of the novel conjugate therapeutic interest and potential clinical impact will be assessed with cell viability assays on tumor and non-tumor cell lines. The conjugate toxicity will be tested on breast cancer and leukemia cell lines and compared to the one of free MTX. A toxicity at least equivalent or higher is expected from the drug conjugate. To test its specificity towards cancer cells, ANXA5-MTX conjugate toxicity will also be assessed on healthy endothelial cell lines, and no toxicity is expected.

MATERIALS AND METHODS

Materials

Annexin A5 recombinant production: pET-30 Ek/LIC vector was purchased from EMD Chemicals (Billerica, MA). Tryptone, yeast extract, and kanamycin monosulfate were obtained from Alfa Aesar (Haverville, MA). Sodium hydroxide, potassium chloride, and sodium chloride were from VWR inc (Radnor, PA). Bovine serum albumin (BSA), Triton X-100, isopropyl β-D-thiogalactopyranoside (IPTG) and, N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride were from Sigma-Aldrich (St Louis, MO). HRV-3C protease was from © Thermo Fisher Scientific (Waltham, MA). Sodium phosphate was from Mallinckrodt Chemicals (Phillipsburg, NJ). The 14 Da dialysis membranes were from Spectrum Laboratories (Rancho Dominguez, CA). His-trap columns were from GE Healthcare (Chicago, IL). HPLC grade ethanol was from Acros Organics (Waltham, MA). HRV-C3 protease was from © Sino Biologics (Wayne, PA). Bradford reagent, 1X Laemnli buffer and 4-20% 10-gradient gels were bought from BioRad (Hercules, CA).

Annexin A5-methotrexate conjugate production: Methotrexate was purchased from TCI America (Portland, OR). Dimethyl sulfoxide (DMSO) was bought from Sigma-Aldrich (St Louis, MO). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased form VWR (Radnor, PA).

Cell culture and in vitro assays: Murine breast cancer cells 4T1 (ATCC® CRL2539™) and EMT6 (ATCC® CRL2755™), leukemia cells P388D1 [P388 D1] (ATCC® CCL46™), human endothelial HUVEC cells (ATCC® CRL1730™), VCBM, Endothelial Cell Growth Kit PCS-100-040, RPMI-1640 medium, Waymouth's MB 752/1

Medium, L-glutamine 200 mM, Dulbecco's Modified Eagle's Medium, horse serum, Ham's F-12K medium, and heparin were from ATCC (Manassas, VA). Human endothelial HAAE-1 cell line was purchased from Coriel Cell Repositories® (Camden, NJ). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Antibiotics penicillin and streptomycin were bought from Invitrogen (Grand Island, NY). 0.2 µm sterile filtration membranes and TrypanBlue were from VWR (Radnor, PA). Alamar Blue reagent was purchased from © Sigma-Aldrich (St Louis, MO).

Methods

Production of recombinant annexin A5

The pET-30 EK/LIC/ANXA5 plasmid encoding annexin A5 was previously constructed in this lab by the PhD student Naveen Palwai[93]. The bacterial strain *E. coli* BL21(DE3) had been transformed to contain this plasmid and is cultured for the recombinant production of ANXA5.

BL21(DE3) *E.coli* is first introduced in a preculture of LB medium overnight before to be added to the 1 L culture medium containing 35 µg/mL of kanamycin. The bacteria cells are cultured at 37°C with a 200 rpm shaking. The OD of the culture medium is regularly monitored with a spectrophotometer at 600 nm, as an indicator of the bacterial growth. Once an optical density of 1.2 is reached, the culture media is induced by the addition of 238 mg of isopropyl β-D-thiogalactopyranoside (IPTG) to induce the constitutive expression of ANXA5. The induction step lasts 19 hours, and the cells are cultured at 25°C to lower the overall protein expression and promote the major production of ANXA5. The next step of the production is to centrifuge the cell culture at 4000 g for 15 minutes to collect the bacterial cells and remove the media. The pellet containing the

cells is then resuspended in a sonication buffer, and the suspension is sonicated 30 seconds at 4.5 W. This sonication is repeated four times for a total sonication time of 2.5 minutes on power level 4. The sonication step aims at breaking the cells membranes to release the produced ANXA5 from the cytosol. A centrifugation step at 12 000 g for 10 minutes is then necessary to separate the soluble proteins fraction from the cell debris and other insoluble components. The supernatant containing the proteins is then retrieved and is purified using a Ni^{2+} chromatography His-trap column. The ANXA5 is produced in the *E. coli* cells with a His-TAG that have an affinity with the Ni^{2+} ions of the column. With the use of the appropriate washing buffer and elution buffers, the protein fraction obtained from the centrifugation is eluted in the column in order to trap the ANXA5-His-TAG in the column and eliminate the undesired non tagged proteins. The elution buffer containing a high concentration of imidazole will release the trapped ANXA5-His-TAG from the column. ANXA5 is then collected and cleaved overnight by adding 10 U of HRV-3C protease per mg of ANXA5. The protease will cleave the C-terminal His-TAG of the protein. The protease also has a C-terminal His-TAG. Thus, the cleaved ANXA5 will be eluted again in the Ni^{2+} chromatography column that will trap the protease and release the cleaved protein. The obtained ANXA5 solution is then dialyzed against 2 L of phosphate buffer saline (PBS) to remove the remaining Imidazole and detergent Triton-X present in the washing and elution buffer. An SDS-PAGE will confirm the successful production and purification of ANXA5 by observing a single band between 36 and 37 kDa. If not used in the next 12 hours, the ANXA5 will be flash frozen in liquid nitrogen and stored at -80°C.

Elaboration of the novel methotrexate conjugation to annexin A5

Methotrexate has several aromatic cycles in its chemical structure and is photosensitive. To preserve its biological properties and its absorbance abilities, all the following experiments containing methotrexate included a protection from light with foil. The preparation of the ANXA5-MTX conjugate was first designed like the one of chlorambucil conjugation to annexin A5, as the chemistry of the conjugate was very similar[90,91]. The first step is the esterification of methotrexate carboxylic functions with EDC to form the unstable O-acylisourea. This intermediate is very reactive towards amine functions to form amide covalent bonds but is also sensitive to hydrolysis. To do so, 3 mg of methotrexate, for instance, are dissolved in 3 mL of PBS. A 10-molar excess of EDC is added, and the solution is vortexed for 5 minutes and strongly stirred for 15 minutes. Then, a 7-molar excess of sulfo-NHS is added to increase the solubility of methotrexate ester and to limit the hydrolysis of MTX ester by pushing the reaction sense towards the formation of the amide bond. EDC being reactive with all carboxylic acids, to limit the formation of undesired cross-linked proteins, 2 µL of β-mercaptoethanol are added to quench the excess of EDC. The methotrexate sulfo-NHS ester solution is then titrated to a pH of 7.4 before being added dropwise to the annexin A5 PBS solution. The protein solution is gently stirred while adding the drug to promote the solubilization and reaction. After 12 hours of gentle stirring at 4°C, the ANXA5-MTX solution is centrifuged at 12 000 g for 10 minutes to remove the unconjugated MTX, precipitated ANXA5, and the other undesired chemicals (EDC, NHS, β-mercaptoethanol). Finally, the conjugate undergoes an overnight dialysis against 2 L of PBS at pH 7.4 to remove the unconjugated methotrexate still soluble.

Characterization of the novel annexin A5-methotrexate conjugate

Absorbance spectroscopy for concentration determination

The first step in characterizing the ANXA5-MTX conjugate is determining both annexin A5 and methotrexate concentrations. The repeated dialysis steps at the end of the conjugation ensure that the remaining methotrexate molecules are conjugated and the ratio of the two concentrations will give the loading ratio of MTX per ANXA5.

As the pterin moiety of methotrexate strongly absorbs in the UV-visible spectrum through its heteroaromatic chromophore cycle, the absorbance peak of methotrexate at 370 nm will be used to establish an absorbance standard curve[20]. The absorbance peak at 370 nm was preferred over the ones at 254 nm and 300 nm because it overlaps the least with protein absorbance at 280 nm. A minimum mass of 2 mg of methotrexate, to limit the uncertainty of the scale, is dissolved in PBS. The primary solution will undergo serial dilutions to obtain the desired range of concentrations of the drug (10 to 180 µM). The absorbance of the different samples will be measured at 370 nm in triplicate with a spectrophotometer to obtain a linear correlation between methotrexate concentration and absorbance. For the establishment of the standard curve, only the concentrations giving an optical density less than 1 will be considered, ensuring the linearity of the curve.

The annexin A5 concentration dosing is assayed with the Bradford protein assay. When exposed to proteins, the Bradford reagent acquires a blue color, and its absorbance at 590 nm is proportional to the protein concentration. The Bradford protein assay being a standardized assay for all proteins, bovine serum albumin is used to establish a standard curve of absorbance for proteins, applicable to measure annexin A5 concentration.

The contributions of the absorbance of MTX to the Bradford assay at 595 nm and the one of annexin A5 at 370 nm were checked.

SDS-PAGE analysis

Once both annexin A5 and methotrexate concentrations were calculated and the loading ratio of the drug conjugate determined, the successful conjugation of the antimetabolite drug to annexin A5 was confirmed by SDS-PAGE. The samples are mixed with 50% 1X Laemnli running buffer with 5% β-mercaptoethanol to break the disulfide bounds and heated in boiling water to get the proteins in their linear form to standardize their migration. The samples are then loaded in a 4-20% 10-gradient gel, migrate in a Tris-glycine-SDS running buffer under 170 V for 30 minutes, stained with Imperial stain, and washed with DI water. Despite denaturing the migrating proteins samples due to the presence of sodium dodecyl sulfate and β-mercaptoethanol, the expected covalent amide bond between MTX and ANXA5 will be preserved. The conjugate should display a higher molecular weight than the free ANXA5, which will be confirmed by a higher band of the free ANXA5 than the ANXA5-MTX conjugate on the gel. Moreover, the SDS-PAGE results will also indicate the purity of the conjugate. Since ANXA5 has both primary amine and carboxylic acid functions in its structure, the EDC mediated conjugation can lead to the formation of ANXA5 cross-linked by-products, which are undesired because their binding to PS could be altered. The analysis of the ANXA5-MTX conjugate allows for an estimate of the extent of this cross-linked species and for the general protein purity in the final conjugate solution.

pH kinetic release assay

The stability and kinetic profile of the drug conjugate was studied in physiological conditions. While the covalent amide bond is strong and stable, it is known that pH and

temperature can influence its chemical stability and possibly lead to a cleavage of the conjugate and a release of methotrexate. The specificity of the therapeutic conjugate relies on the stability of this bond. ANXA5-MTX conjugate (3.2 mg in 2 mL) containing 150 µM of MTX was put in a MWC0 14 000 Da dialysis bag in 50 mL of dialysis buffer at 37°C under mild agitation. The concentration of MTX in the buffer was periodically measured spectrophotometrically, by withdrawing 5 mL of buffer at predetermined time intervals and replenishing the samples with an equal volume of fresh medium. This provides a curve of cumulated released MTX concentration through time. The assay was conducted in two different pH buffers: the physiological extracellular pH of 7.4 and the tumor extracellular pH of 6.6. A positive control was used with a dialysis bag containing free MTX at the same concentration as the ANXA5-MTX conjugate.

Storage conditions stability assay

Because of the possible effect of extended times in extreme temperatures on protein viability, storage of the ANXA5-MTX conjugate may have an impact on the stability and conservation of the therapeutic agent. To assay the storage conditions of the conjugate, samples stored for different times at -80°C and 4°C were characterized and compared to their initial values of ANXA5 and MTX concentrations. The samples are dialyzed for 2 hours against 2 L of PBS to remove the unconjugated methotrexate and centrifuged at 3000 g for 5 minutes to remove the precipitated and insoluble annexin A5 proteins.

Cell culture conditions

For all described murine and human cell lines, the cells were cultured in an incubator at 37°C with 5% CO_2 in T-75 flasks. The sub-cultures were continuously passaged when a confluency of 70% was reached to maintain the cell populations in an exponential phase. The cryopreservation was ensured by adding 5% of DMSO and putting the cells overnight

in isopropanol before storing them at -80°C in the cryotank. All treatment samples were sterile filtered using 0.2 µm membranes before being administered to cells.

Cell counting

The adherent cell culture to count is trypsinised with 2 mL of trypsin-EDTA for 5 minutes at 37°C. Culture medium (8 mL) is added and the cell suspension is centrifuged at 500 g for 5 minutes to remove the trypsin. The supernatant is discarded, and the pellet is resuspended with 10 mL of medium. Then 100 µL of the final suspension is retrieved and counted with a hemocytometer using TrypanBlue exclusion cell viability reagent. For the non-adherent P388 cell culture, the cell counting was performed by directly collecting 100 µL from the cell culture flask.

Breast cancer cell lines

Two metastatic triple negative breast cancer murine cell lines were used. Both are highly tumorigenic and invasive. 4T1 (ATCC® CRL2539™) is a mammary gland carcinoma cell line that is an animal model for stage IV of human breast cancer. It is cultured in RPMI-1640 supplemented with 10% of FBS and 1% of Anti-Anti (antimycotic/penicillin/100 $U.mL^{-1}$).

EMT6 (ATCC® CRL2755™) is a mammary carcinoma cell line and is cultured in Waymouth's MB 752/1 medium supplemented with 15% of FBS, 2 mM of L-glutamine and 1% of Anti-Anti.

Leukemia cell line

P388 D1(ATCC® CCL46™) is a murine lymphoblastic leukemia non-adherent cell line. P388 cells are malignant monocytes grown in suspension that differentiate into adherent macrophages. They are grown in DMEM medium supplemented with 10% horse serum

and 1% of Anti-Anti. To maintain the cell culture in an exponential phase, the P388 culture was regularly counted and maintained to a concentration of $2 – 5.10^5$ cells/mL.

Healthy endothelial cell lines

Two human non-tumorigenic endothelial cell lines were chosen to assay the cancer cells specificity of the ANXA5-MTX conjugate. As the therapeutic agent will be considerably exposed to the endothelium of blood vessels during its transport in the blood stream, the toxicity of the novel drug conjugate on endothelial cells will provide a relevant estimation of its off-target potential and specificity towards tumor cells.

HUVEC (ATCC® CRL1730™) is a cell line based on human umbilical vein endothelium. It is cultured with VCBM medium supplemented with the Endothelial Cell Growth Kit PCS-100-040 containing 10 mL of FBS, 1% Anti-Anti, 25 mL L-glutamine, 0.5 mL ascorbic acid, 0.5 mL heparin sulfate, 1 mL of bovine brain extract, 0.5 mL of rhEGF, and 0.5 mL of hydrocortisone.

HAAE-1 (Coriel Cell Repositories®) is a cell line from the human abdominal aorta endothelium. This cell line is cultured with the Ham's F-12K medium supplemented with 0.1 mg mL^{-1} heparin, 0.03 mg mL^{-1} endothelial cell growth supplement, 10% fetal bovine serum, and 1% of Anti-Anti.

Alamar Blue cytotoxicity in vitro assay

The Alamar Blue® cell viability reagent is a cytotoxicity assay relying on the reduction of the poorly fluorescent blue indicator dye resazurin into the pink highly fluorescent resorufin[94]. The Alamar Blue test was chosen because more convenient to realize: the reagents have a good solubility in water, and no steps of dissolution or resuspension are needed, therefore reducing the variability of the volumes of the wells before the reading

step. The reduction of resazurin is performed by the mitochondrial electron transport enzymes, considered here as a life indicator. By measuring the fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 590 nm, the cell viability can be quantified and standardized with a control condition. The Alamar Blue assays was conducted on 96-well plates with a cell seeding and incubation times that depend on the cell line (detailed protocol is provided in the appendix). When treated, the cells receive fresh media containing 2 mM of Ca^{2+} and different concentrations of treatment (free MTX and ANXA5-MTX). At the end of the treatment time, the media of the plate was replaced with fresh new media containing 10% of Alamar reagent. A triplicate of unseeded wells only containing culture medium was used to remove the noise of the overall fluorescence. Several triplicates of untreated wells will serve as positive control for the 100% cell viability. To limit the variability of the wells volumes due to evaporation in the incubator, the outer wells of the plates were not used and instead filled with 200 µL of PBS. The treated wells fluorescence was divided by the positive control one to give a percentage of cell viability. The IC50 values (inhibitory concentration at 50% viability), that is the main cytotoxicity indicator for a drug, was calculated with the IC50 calculator of the website *AAT Bioquest*.

Statistical Analysis

Cytotoxicity analysis of IC50 data in tumor cells assays was performed using a Student t-test, and a one-way ANOVA with a Tukey-Kramer multiple comparisons test was performed to analyze the non-tumor cells viability assays using R Studio 2020 software. Error is graphically represented as standard error (SE) bars calculated with Excel 2019.

RESULTS

Protocol development for the novel conjugation of methotrexate to annexin A5

Despite of the solubility of around 1 mg/mL of MTX in PBS, the formation of the O-acylisourea intermediate by esterification of MTX carboxylic acid with EDC imide bond considerably decreased the solubility of the drug in PBS and limited the conjugation to ANXA5 due to high precipitation and loss of MTX. The adding of sulfo-NHS, which is supposed to enhance the solubility of MTX, did not have any effect. The first step of protocol optimization was to dissolve MTX and EDC in DMSO, in which the drug solubility is higher (3 mg/mL). MTX has a higher solubility in DMF (14 mg/mL), but the use of this solvent requires an extra step of decantation due to the formation of urea precipitates[39]. To enhance the linking of MTX to EDC, a 15 minute-water bath at 50°C was found to be efficient[40–42]. As DMSO can cause an alteration of proteins at high concentrations leading to crystallization, precipitation, and denaturation, it was made sure that the DMSO fraction would not exceed 10% of the total reaction volume[95]. Not adding the quencher β-mercaptoethanol was found to contribute to the successful binding of MTX to ANXA5, suggesting that the conjugation reaction would still occur during the dialysis step. Also, it was found that slowly adding the esterified MTX to the ANXA5 solution in PBS dropwise while regularly stirring the vial made the solution clearer and less turbid, and highly increased the ANXA5 and MTX yield by limiting their precipitation. The pH of the solution was carefully adjusted to 7.4 ± 0.02 while adding the DMSO solution of MTX and EDC to preserve the biological function of ANXA5. The reaction time between ANXA5 and MTX was shortened from 12 hours to 30 minutes and switched to room temperature instead of 4°C to optimize the kinetics of the reaction.

To maximize the contact time between ANXA5 and MTX, it was chosen to centrifuge the conjugate solution only after dialysis and not before. The centrifuge parameters were also optimized to be lowered to 3000 g for 5 minutes to remove the precipitated ANXA5 and cross-linked chemicals that were not removed during dialysis, while preserving a maximum of soluble conjugates in the clear supernatant. The final optimized novel protocol of conjugation of methotrexate to annexin A5 is available in the appendix.

Establishment of methotrexate absorbance standard curve

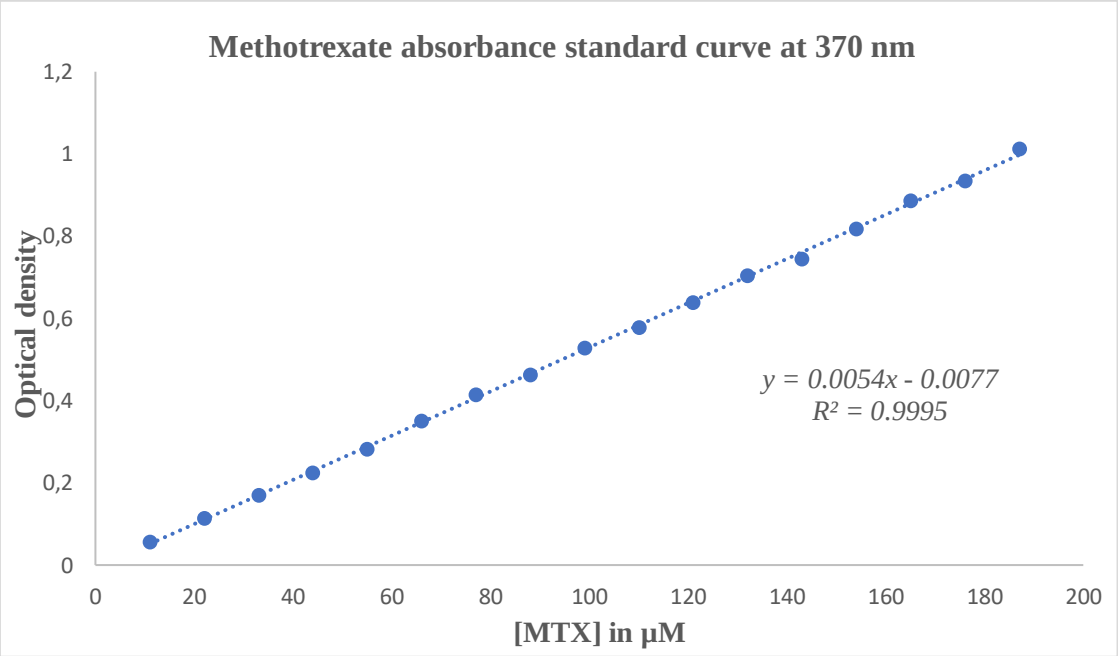

Fig. 7 Standard curve of methotrexate abosrbance at 370 nm in PBS. The data points were obtained from triplicate absorbance values of samples from 10 to 185 µM of MTX using a spectrophotometer. .

Methotrexate has the characteristic to have an absorbance peak at 370 nm. This physical property of the molecule is used to establish the standard curve that will allow for an easy and reliable way of measuring its concentration in a solution. The absorbance at 370 nm has been measured over a range of concentrations of MTX in triplicate in PBS from 10 to 185 µM (*Figure 7*).

Gel electrophoresis image of the ANXA5-MTX conjugate

Figure 8 shows the SDS-PAGE gel migration of the free annexin A5 and the conjugated protein to methotrexate. From this gel, we can see that the conjugate has a slightly higher molecular weight than the free protein, confirming the successful EDC/NHS mediated conjugation, that led to the formation of a stable amide bond between the carboxylic functions of methotrexate and the amine functions of annexin A5. To get the exact loading ratio of the conjugate, the MTX concentration of the final conjugate solution is measured with the standard curve and the number of MTX per ANXA5 is deduced. Repeated steps of dialysis at the end of the conjugation allows for the complete removal of unconjugated methotrexate and ensures that the drug concentration dosed is the conjugated one. It is recommended by Thermo Fisher to couple the reaction of esterification with sulfo-NHS to promote the reaction sense to the formation of the stable amide bond of the final conjugate and limit the hydrolysis of the MTX-EDC intermediate component. The loading ratio, number of conjugated MTX molecules per annexin A5, was calculated by determining the ratio of each concentration. As observable in *Figure 8*, the conjugate in the lane 3 with a loading ratio of 4 with the single EDC coupling method is slightly smaller than the conjugate based on EDC-NHS coupling method with a higher loading ratio of 7. However, if sulfo-NHS does increase the loading ratio of the ANXA5-MTX conjugate, it also causes a higher formation of cross-linked proteins and a more smeared band on the SDS-PAGE, reflecting a higher heterogeneity of the number of MTX per annexin A5 in the final conjugate solution. Therefore, it has been chosen to carry the following conjugation strategies only with the EDC coupling that offers a lower loading ratio but a sharper band and a more homogenous ANXA5-MTX conjugate binding.

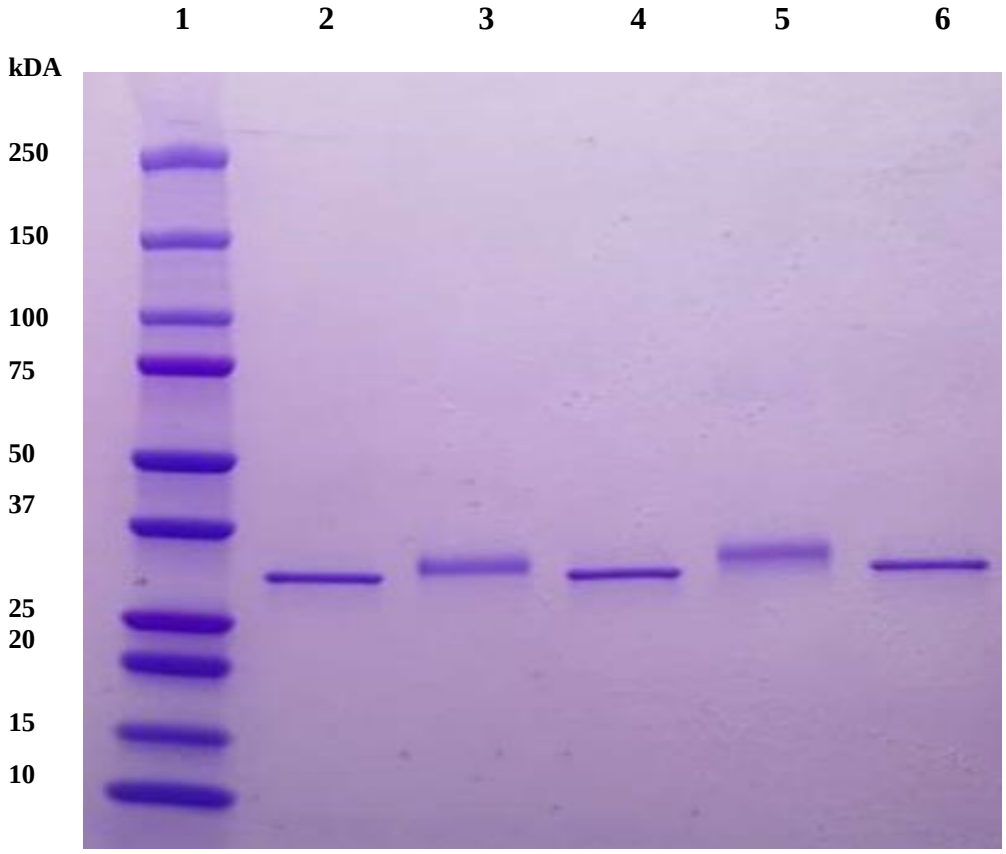

Fig. 8 SDS-PAGE of free ANXA5 and ANXA5-MTX conjugates. Molecular ladder (1) ; Purified and cleaved annexin A5 (lanes 2, 4, 6); Annexin A5-methotrexate conjugate based on EDC coupling with a loading ratio of 4 (lane 3); Annexin A5-methotrexate conjugate based on EDC-NHS coupling with a ratio of 7 (lane 5).

Release Kinetic assays of the ANXA5-MTX conjugate

Figure 9 shows the cumulative release of methotrexate from ANXA5-MTX conjugate in PBS at 37°C, at pH 7.4 and 6.6. A free MTX sample was added to the experiment as a positive control. All three samples had the same concentration of drug at the beginning of the assay (150 µM). We can see that almost the totality of free MTX was released in approximately 3.5 hours, while the conjugated methotrexate released around 30% of its methotrexate total amount in 48 hours, for both pH values of 7.4 and 6.6. These release kinetic assays confirm that the amide bond between MTX and annexin A5 obtained from

the EDC/NHS conjugation is not lysed by a 48-hour exposure to physiological conditions (37°C, pH of 7.4). Moreover, this assay also shows that the acidic tumoral environment (pH of 6.6) does not induce an early cleavage of the conjugate, supporting the hypothesis that conjugated methotrexate will be internalized in the cancer cells through annexin A5 binding pathway to phosphatidylserine. It also reinforces the specificity of ANXA5-MTX conjugate towards cancer cells by showing a low release of the drug in the extracellular media, that could potentially damage healthy cells.

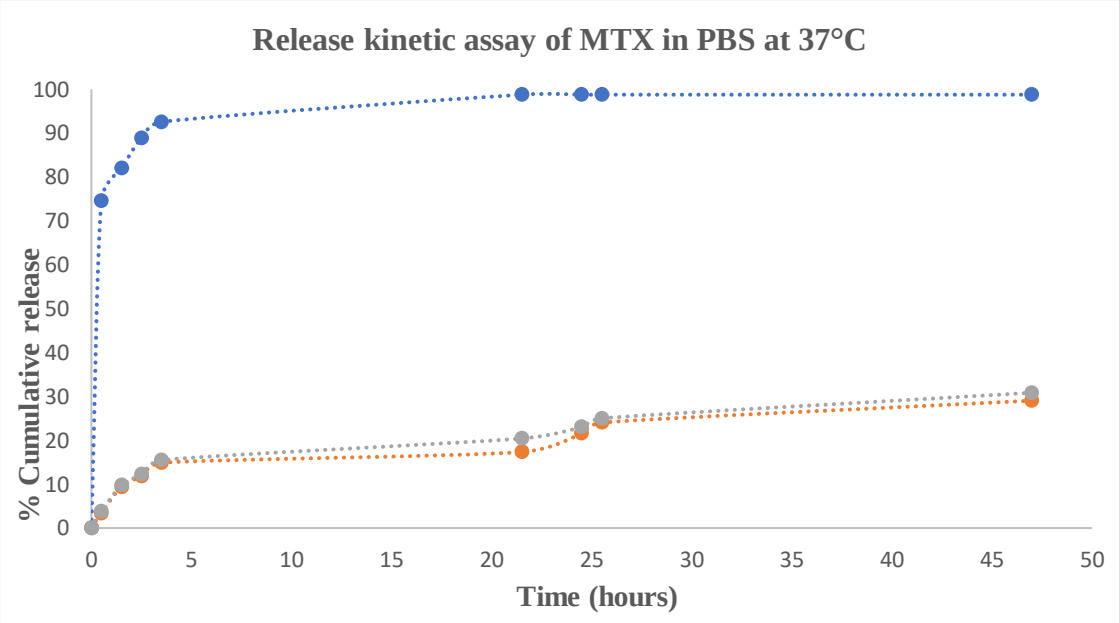

Fig. 9 Release kinetic assay of free MTX and ANXA5-MTX conjugate. Cumulative release of MTX in free MTX (blue), ANXA5-MTX conjugate at pH 7.4 (orange) and ANXA5-MTX conjugate at pH 6.6 (grey), in PBS at 37°C. Data points were obtained from triplicate absorbance values at 370 nm using a spectrophotometer.

Storage stability assay of ANXA5-MTX conjugate

To evaluate the impact of the storage conditions at 4°C and -80°C on the ANXA5-MTX conjugates, the final concentrations of annexin A5 and methotrexate are measured and the percentages from the initial concentrations are deduced and used as an indicator of preservation. Before doing so, the samples are dialyzed for 2 hours in PBS to remove the

potentially unconjugated methotrexate. The samples are then centrifuged at 3000 g for 5 minutes to discard all precipitated annexin A5.

Stability assay of ANXA5-MTX conjugate at 4°C (fridge)

ANXA5-MTX Conjugate	Time of storage (4° C)	% of initial [ANXA5]	% of initial [MTX]
A	4 days	90.5 %	82.5 %
B	46 days	80.6 %	46.0 %
C	55 days	59.9 %	55.2 %
D	63 days	31.4 %	52.4 %
E	4 months	00.0 %	33.3 %

Table. 1 Table of percentages of final concentration of annexin A5 and methotrexate in conjugates, stored for different times at 4°C. Measurements were done on five different conjugates after 2 hours of dialysis in PBS and 5 minutes of centrifugation at 3000 g.

Stability assay of ANXA5-MTX conjugate at -80°C (freezer)

ANXA5-MTX Conjugate	Time of storage (-80° C)	% of initial [ANXA5]	% of initial [MTX]
F	38 days	98.7 %	89.8 %
G	55 days	98.3 %	65.4 %
H	116 days	82.4 %	41.5 %
I	116 days	96.1 %	72.5 %
J	4 months	99.2 %	61.5 %

Table. 2 Table of percentages of final concentrations of annexin A5 and methotrexate in conjugates, flash frozen in liquid nitrogen and stored for different times at -80°C. Measurements were done on five different conjugates after 2 hours of dialysis in PBS and 5 minutes of centrifugation at 3000 g.

Results of *Table 1* show that samples stored at 4°C have a satisfactory annexin A5 conservation up to the first 6 weeks of storage (80.56% of preserved annexin A5 after 46 days at 4°C). However, the number of viable proteins decreases considerably after 3 months (31.4% after 63 days) and is undetectable on a Bradford assay after 4 months.

The decrease in methotrexate concentration through time is attributable to both annexin A5 carrier loss and amide bond cleavage. The storage assays result at -80°C (*Table 2*) show a significantly better conservation of annexin A5 when flash frozen in liquid nitrogen (99.2% of initial annexin A5 amount is preserved after 4 months of storage at -80°C). However, the cryopreservation still induces a certain loss of conjugated methotrexate: 41.54% of initial conjugated drug concentration was measured after 116 days of storage and 61.5% of another conjugate after 4 months. Given the high conservation of ANXA5, the loss of conjugated MTX is mostly due to a cleavage of the amide bond of the drug conjugate induced by the temperature changes.

Cytotoxicity assays results

The therapeutic impact of the novel ANXA5-MTX conjugate relies on two main conditions. The drug conjugate must first be able to deliver an antineoplastic effect that is at least as efficient as the one delivered by the free drug on cancer cells. Also, the conjugate must display a certain specificity towards cancer cells and is expected to deliver a non-significant toxicity when exposed to healthy cells that do not externalize phosphatidylserine. To analyze and compare the toxicities of the treatments on cancer cells, the parameter IC50 will be calculated and exploited. In this case, the IC50 is the concentration of drug necessary to induce 50% of cell death, meaning that a lower IC50 indicates a higher potency of the drug. The following in vitro assays present the cell viability results on two healthy human endothelial cell lines and three murine tumor cell lines when treated with free and conjugated MTX.

Non-tumor cell viability assays

HAAE-1 cell line

Figure 10 shows the toxicity of the free and conjugated methotrexate on healthy human HAAE-1 endothelial cells, that are extracted from the abdominal aorta. We can see that the ANXA5-MTX conjugate does not cause a significant cell mortality, whereas the free MTX induces a cell mortality that goes up to 45% at 30 µM. The HAAE-1 cell line has already been used as a healthy model of cells[96], that do not externalize the phosphatidylserine when a confluency close to 100% is reached. Methotrexate is a chemotherapeutic that is mostly active on cells in phase S; the high confluency of the cells during the Alamar Blue assay explains why methotrexate cell toxicity is lower than in the tumor cell lines. These results support the specificity of the ANXA5-MTX conjugate towards cancer cells via phosphatidylserine binding.

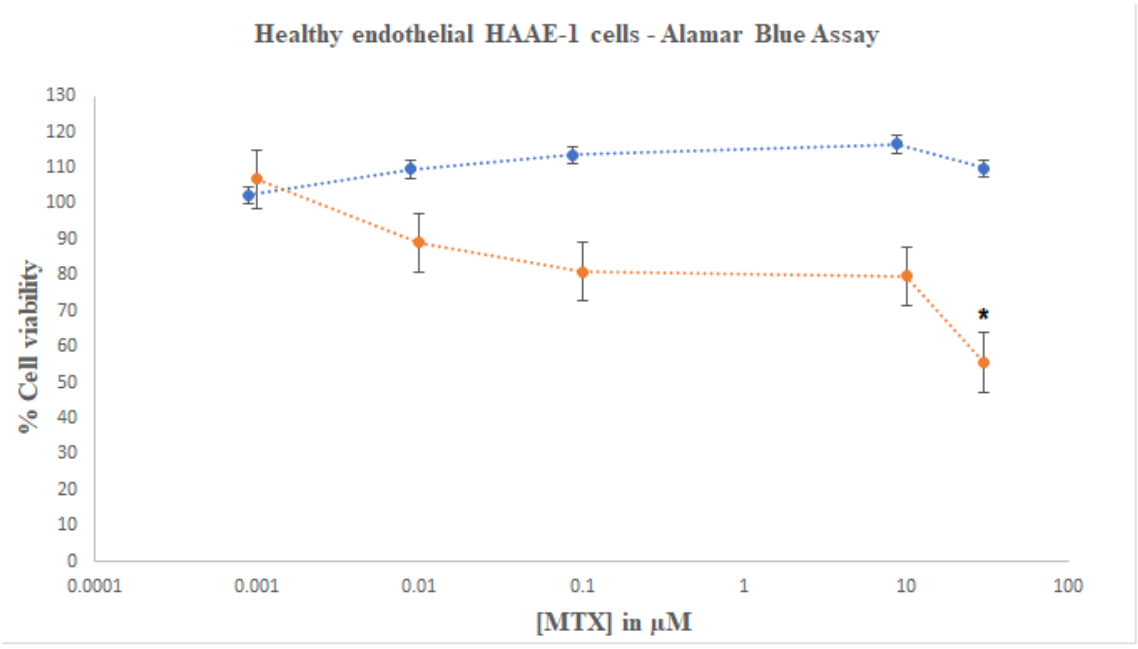

Fig. 10 Alamar Blue assay on human healthy endothelial HAAE-1 cells after 48 hours of treatment. Free methotrexate (orange) and ANXA5-MTX conjugate with a drug ratio of 6 (blue). The cells were seeded at 10,000 per well with 200 µL of Ham's F-12K media, each, then treated 48 hours after when a confluency of 90% was reached. Data is presented

as mean ± SE (n = 3). Statistical significance of conjugate versus MTX denoted by * ($P < 0.05$).

HUVEC cell line

To strengthen the last results on the HAAE-1 cell line, a similar cell viability assay was conducted on the HUVEC cell line. HUVECs are non-tumor cells cultured from human umbilical vein endothelium. Like HAAE-1, they are known to not externalize the phosphatidylserine when a confluency close to 100% is reached. The results of the cell viability assay clearly indicate that while free MTX induces a cell mortality that reaches 51% with a 44 µM drug concentration, the conjugate does not induce a statistically significant cell mortality at equivalent concentrations (*Figure 11*). These results are consistent with the HAAE-1 cell results and confirm that the ANXA5-MTX conjugate does not induce a significant cell mortality on healthy endothelial cells.

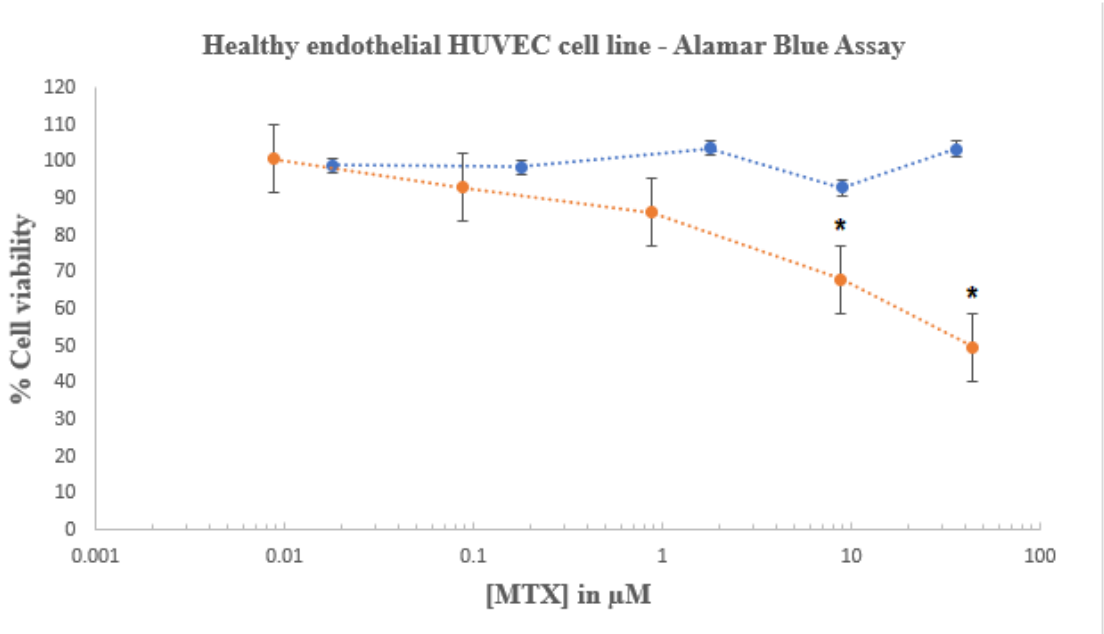

Fig. 11 Alamar Blue assay on human healthy endothelial HUVECs cells after 48 hours of treatment. Free methotrexate (orange) and ANXA5-MTX conjugate with a drug ratio of 6 (blue). The cells were seeded at 10,000 per well with 200 µL of VCBM media, each, then treated 48 hours after when a confluency of 90% was reached. Data

is presented as mean ± SE (n = 3). Statistical significance of conjugate versus MTX denoted by * ($P < 0.05$).

Tumor cells viability assay

MTX is an FDA approved chemotherapeutic drug for a wide range of malignancies including breast carcinoma, acute lymphoblastic leukemia, and non-Hodgkin lymphoma. Proving that the toxicity of ANXA5-MTX conjugate is at least equivalent to the one of the free MTX would establish the therapeutic impact of the novel drug conjugate on already FDA approved malignancies. The following in vitro results present the cell viability assays of one non-adherent P388 cell line and two adherent 4T1 and EMT6 tumor cells.

P388 cell line

The P388 cell line consists of tumorigenic monocytes and macrophages that are commonly used as a model for leukemia and lymphoma studies[97–101]. In *Figure 12,* we can see that the two cell viability curves are close, indicating that the free MTX and ANXA5-MTX conjugate have comparable toxicities to P388 cells. The experiment was repeated two more times, and a Student test was conducted on the two groups of three IC50s of the three experiments. The resulting calculated mean IC50 are respectively 0.21 ± 0.12 µM for free MTX and 0.11 ± 0.01 µM for ANXA5-MTX. A P value of 0.34 was obtained, confirming that ANXA5-MTX conjugate has a toxicity towards the leukemia and lymphoma cell model that is statistically equivalent to the one delivered by the free drug. These results are encouraging and show a therapeutic potential in improving methotrexate clinical treatment in acute lymphoblastic leukemia and non-Hodgkin lymphoma.

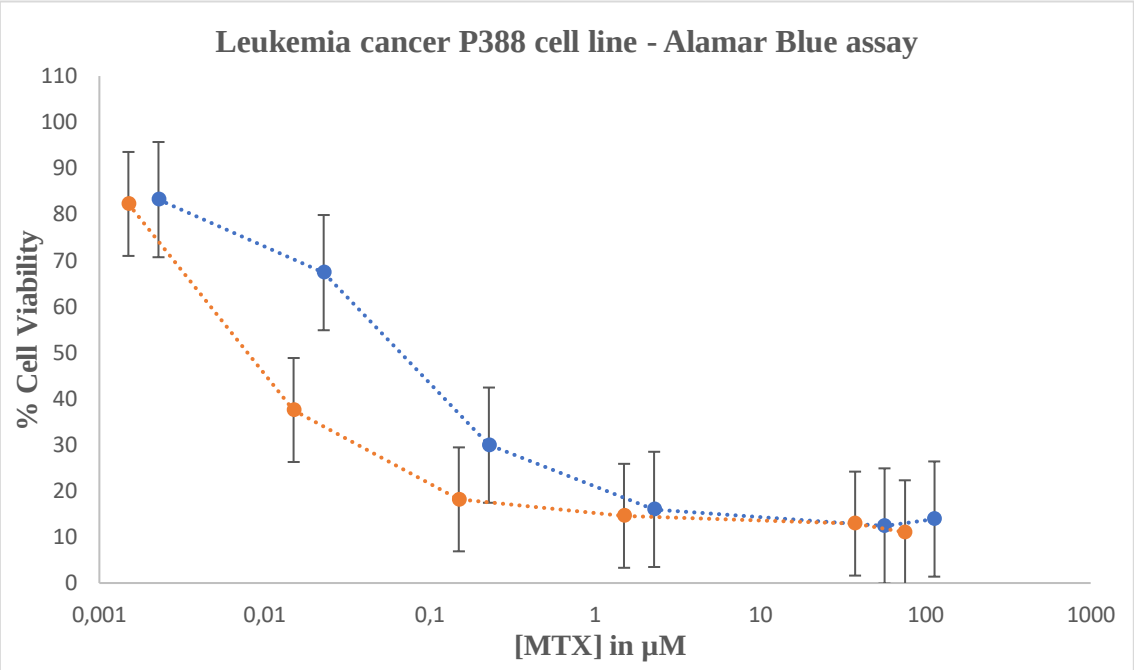

Fig. 12 Alamar Blue assay on murine leukemia P388 cells after 48 hours of treatment. Free methotrexate (orange) and ANXA5-MTX conjugate with a drug ratio of 6 (blue). The cells were seeded at 20,000 per well with 200 µL of DMEM media. Data is presented as mean ± SE (n = 3).

EMT6 cell line

EMT6 cell line has recently emerged as a valuable pre-clinical model of study for triple negative breast cancer[102–108]. In *Figure 13*, we can see the proximity of the sigmoid cell viability curves of free and conjugated MTX. The experiment was repeated two more times, and a Student test was conducted on the two groups of three IC50s of the three experiments. The resulting calculated mean IC50 are respectively 0.03 ± 0.00 µM for free MTX and 0.18 ± 0.06 µM for ANXA5-MTX. A P value of 0.18 was obtained. The ANXA5-MTX conjugate delivers a toxicity on EMT6 triple negative breast cancer cells that is statistically equivalent to the free MTX.

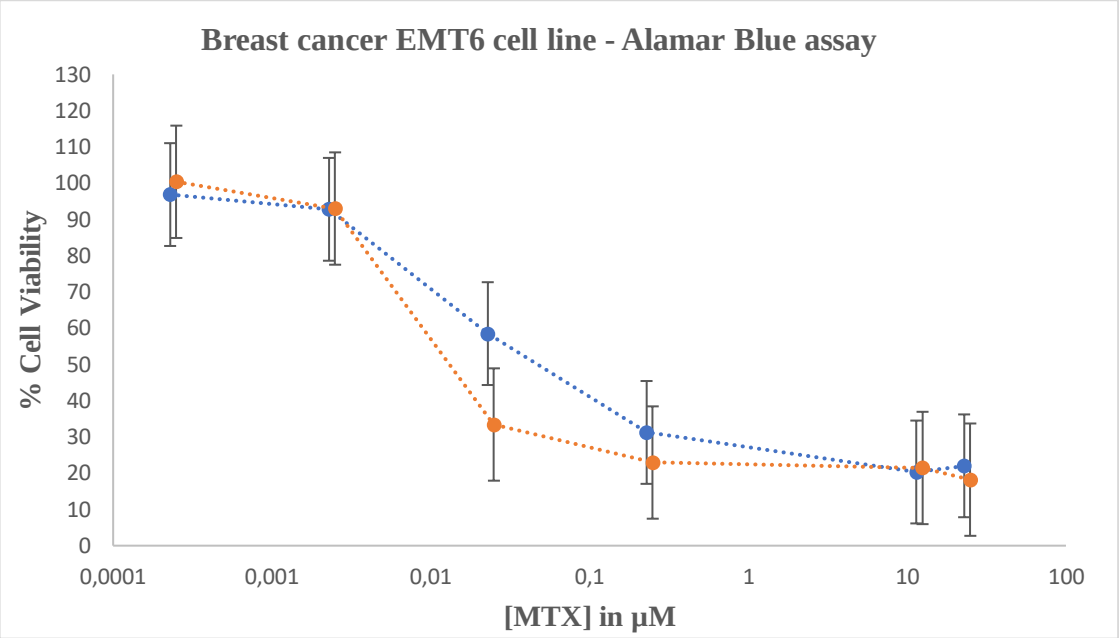

Fig. 13 Alamar Blue assay on murine breast cancer EMT6 cells after 48 hours of treatment. Free methotrexate (orange) and ANXA5-MTX conjugate with a drug ratio of 6 (blue). The cells were seeded at 5000 per well with 200 µL of Waymouth medium. Data is presented as mean ± SE (n = 3).

4T1 cell line

4T1 cells are a murine model for stage IV of human triple negative mammary gland carcinoma. In *Figure 14*, we can see again the proximity of the cell viability curves of free MTX and ANXA5-MTX. The experiment was repeated two times and a Student test was conducted on the two groups of three IC50s of the three experiments. The resulting calculated mean IC50 are respectively 0.1 ± 0.00 µM for free MTX and 0.38 ± 0.11 µM for ANXA5-MTX. A P value of 0.174 was obtained, confirming the equivalent toxicity of ANXA5-MTX conjugate to free MTX on 4T1 cells.

Despite of not having obtained a drug conjugate with an increased toxicity on cancer cells compared to the free drug, ANXA5-MTX has induced a toxicity statistically comparable to free MTX in both leukemia-lymphoma P388 model cell line and the two triple negative

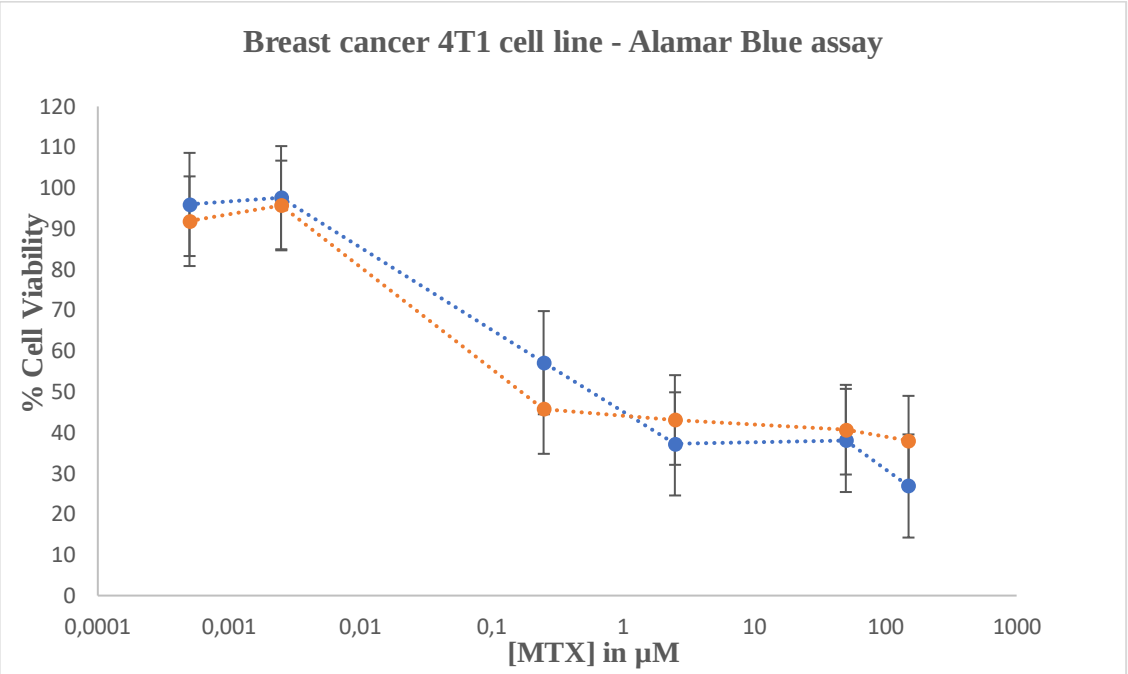

Fig. 14 Alamar Blue assay on 4T1 breast cancer murine cell line after 48 hours of treatment. Free methotrexate (orange) and ANXA5-MTX conjugate with a drug ratio of 6 (blue). The cells were seeded at 5000 per well with 200 µL of RPMI-1640 medium. Data is presented as mean ± SE (n = 3).

breast cancer 4T1 and EMT6 cells. This conclusion shows that the EDC/NHS conjugation chemistry to ANXA5 did not significantly impact MTX antineoplastic function. Significantly, ANXA5-MTX did not deliver a significant cytostatic effect in the two healthy endothelial cell lines tested in this work, confirming the effective targeting of ANXA5 towards phosphatidylserine and the preservation of ANXA5 biological function. The combination of these two conclusions strongly support the therapeutical potential of the novel ANXA5-MTX conjugate to respond to the urgent needs of improving methotrexate formulations in current clinical therapies against cancer.

DISCUSSION

Cancer is one of the most challenging diseases to treat nowadays. The difficulty to find a successful treatment comes from many properties of the tumor cells. Their ability to develop chemoresistance, to promote anarchic angiogenesis in their microenvironment, and metastasis to ectopic sites renders most of current treatments partially or poorly efficient. Leukemia and lymphoma are liquid tumors for which recommended treatments can be chemotherapy, radiation therapy, or bone marrow transplant. Triple negative breast cancer is a type of malignancy that does not express any receptor sensitive to hormonal therapy (estrogen receptors, progesterone receptors or HER2) and which the easiest way to treat is the surgical ablation of the tumor. However, if these malignancies develop metastasis, surgery is no longer efficient as a treatment. Methotrexate is a well-known chemotherapeutic agent approved for the treatment of many malignancies. However, its fast metabolization in the organism considerably limits its efficient delivery to the tumor site. It is known that less than 1% is estimated to reach the tumor site, the rest of the drug fraction being randomly distributed in the body.

The current increase of MTX necessary doses to observe a tumor decline in the clinic are also responsible for many deleterious clinical side effects. Targeted therapies against cancer therefore play a central role in the development of optimum novel anti-tumor treatments. They allow for a higher delivered drug fraction to the tumor site without damaging the non-tumor cells, which lowers the administered doses in patients and limits the development of chemoresistance from the tumor. As presented here, we propose to exploit the property of the protein ANXA5 to bind to phosphatidylserine. Phosphatidylserine is a phospholipid ubiquitously present in eucaryotic cells but is mainly externalized in apoptotic and tumor cells.

By conjugating the chemotherapeutic agent MTX to ANXA5, we design a novel targeted treatment that presents a high therapeutic potential regarding the current clinical needs of chemotherapy. ANXA5-MTX was successfully produced using the standard EDC/NHS coupling and after optimizing different steps of the pre-established conjugation protocol used for chlorambucil conjugation. The novel conjugate was obtained with satisfactory ratios going from 2 to 19 molecules of MTX per ANXA5, confirmed by the higher conjugate bands than the free protein band on the SDS-PAGE. The cytotoxicity assays did not show a higher toxicity of the conjugate compared to the free drug as with the chlorambucil or DM1 conjugate previously elaborated[90,91]. However, ANXA5-MTX was found to have a toxicity statistically equivalent to free MTX on lymphoma P388 cell line and triple negative breast cancer 4T1 and EMT6 cell lines. This proves that the conjugated formulation does not significantly impact MTX cytostatic function.

One of the most probable reasons for not observing a higher toxicity of the conjugate is that methotrexate internalization in the cells is mediated by active ATP-dependent transport via the reduced folate receptor FOLR1 and the folate receptor RFC[21–24]. Indeed, as a folate analog, free MTX is recognized by the cells and undergo an active endocytosis that compete with the ANXA5 internalization through phosphatidylserine. Furthermore, we know that ANXA5 internalization is complex and depends on several factors. The calcium-dependent endocytosis goes through the formation of a trimer of bonded ANXA5 to phosphatidylserine that then assembles to another trimer to form a two-dimensional lattice that will then induce a macropinocytotic endocytosis in the cancer cell. Moreover, the conjugate did not induce any significant cell death on the two tested healthy endothelial cell lines HAAE-1 and HUVEC, highlighting the therapeutic promising potential of the innovative ANXA5-MTX drug conjugate. These assays on healthy cells

prove that the conjugated methotrexate toxicity delivery is mediated by ANXA5 binding to phosphatidylserine, and that the conjugation design prevents the drug to exert its cytostatic function when exposed to healthy cells. However, it is important to confirm the behavior of ANXA5-MTX conjugate on non-endothelial healthy cells such as mammary epithelial cells and healthy monocytes and lymphocytes to strengthen the specificity of the conjugate. Moreover, in vivo assays using mice would give further information on the pharmacokinetics of the conjugated MTX compared to the free one in a living organism. Indeed, several non-targeted MTX designed conjugates such as albumin or nanoparticles showed promising in vivo results because of the lowered blood clearance of the conjugated MTX, its resistance to being metabolized, and the better exposure to the tumor site due to the enhanced permeability effect of tumoral vasculature[18, 20-29].

CONCLUSION

One of the most currently challenging issues of chemotherapy in cancer treatments relies on specifically targeting and carrying the drug to the tumor cells. Annexin A5 presents a high potential regarding this challenge as it can specifically bind to phosphatidylserine and be internalized in the cell. Because phosphatidylserine is externalized only in apoptotic and tumor cells, annexin A5 is a promising candidate for conjugation as a drug carrier. Methotrexate is a folate analog that inhibits the synthesis of nucleotides necessary for DNA replication and protein synthesis. Methotrexate is approved for the treatment of many malignancies, but there is an urgent clinical need for the development of a targeted chemotherapy as the available therapies including methotrexate induce considerable side effects and chemoresistance in the treated patients. Many protein and nanoparticles conjugations have been attempted on methotrexate, but none showed a significant specificity towards cancer cells. In this work, we developed an innovative conjugate of methotrexate to annexin A5 with a satisfactory and stable loading drug ratio. The drug conjugate delivers a toxicity on triple negative breast cancer and lymphoma cell lines equivalent to the one of the free drugs. The ANXA5-MTX conjugate also turned out to not deliver any significant toxicity on the two tested human endothelial cell lines, making it promising for further investigations in *in vivo* mice and clinical trials.

FUTURE DIRECTIONS

Animal model tests of the novel annexin A5-methotrexate conjugate should be conducted to evaluate its potential as a therapeutic. The slower blood clearance and the enhanced microvascular permeability give the conjugate a better pharmacokinetic profile for a higher and specific drug fraction delivered to the tumor site and lower off-target effects due to the lower diffusion of the conjugated drug in systemic tissues.

A catalytic assay should be conducted to analyze the activity of the enzyme DHFR, a hallmark of the cellular toxicity of methotrexate, to allow for a more specific quantification of methotrexate toxicity in cells, and to support the Alamar Blue cell viability assays. A cellular uptake assay using a FITC linkage to ANXA5-MTX would allow for a quantification of the bound conjugate, that would reflect its binding efficacy, but also visualize the conjugate delivery in cells, its specific binding, and internalization with confocal microscopy. For the healthy cell viability assay, assessing the toxicity of the conjugate on human epithelial mammary healthy cells such as MCF-10A would confirm the specificity of the conjugate towards cancer cells. Evaluating the cytotoxicity of ANXA5-MTX conjugate on human breast cancer MCF-7 and MDA-MB 231 would show if the conjugate delivers a satisfactory toxicity on human cancer cells.

Because the ANXA5-MTX conjugate would bind to the vasculature of tumors and therefore cut off the tumor's blood supply, therapy in vivo should be combined with an mTOR inhibitor such as rapamycin, which inhibits the response of the tumor to hypoxia. Previous studies from our lab have shown that an enzyme prodrug targeted to the breast tumor vasculature using ANXA5 gave a significant reduction in the size of breast tumors in mice when rapamycin was added compared to when it was not added[109]. In addition, MTX causes an increase of reactive oxygen species (ROS) amount, which can lead to cell

necrosis and the release of tumor antigens[110]. Therefore, therapy in vivo should also be tested in combination with immune checkpoint inhibitors such as anti-CTLA-4, which we recently have shown to be effective in mice with breast tumors when combined with photothermal therapy of breast tumors that causes tumor antigens to be released (P. McKernan, N. Virani, G. Faria, C. Karch, R. Prada Silvy, D. Resasco, and R. Harrison, Targeted single-walled carbon nanotubes for photothermal therapy combined with immune checkpoint inhibition for the treatment of metastatic breast cancer, Nanoscale Research Letters, accepted for publication, 2020).

ABBREVIATIONS

ECM	Extracellular matrix
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
TNBC	Triple negative breast cancer
MTX	Methotrexate
DHFR	Dihydrofolate reductase
FA	Folic acid
THF	Tetrahydrofolate
dAMP	Deoxyadenosine monophosphate
dGMP	Deoxyguanosine monophosphate
TS	Thymidylate synthase
[MTX]	Concentration of methotrexate
[ANXA5]	Concentration of annexin A5
ANXA5	Annexin A5
ANXA5-MTX	Annexin-A5-methotrexate conjugate
EDC	Ethyldiethylcarbodiimid
Sulfo-NHS	Sulfo-N-hydroxysuccinimid
UV	Ultraviolet
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
DMSO	Dimethyl sulfoxide
DMF	Dimethylformamide
RPMI-1640	Rosewell Park Memorial Institute
VCBM	Vascular cell basal medium
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
HS	Horse serum
OD	Optical density

IC50	Inhibitory concentration at 50%
Anti-Anti	Antibiotic and antimycotic

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APPENDIX

Initial ANXA5-MTX conjugation protocol (10 mg of conjugate)[90,91]

1- Add 1,49 mg of methotrexate in 1,50 mL of PBS (3.29×10^{-3} mmol).

2- Add 100 mg of EDC ($5,21 \times 10^{-1}$ mmol).

The EDC will bind the carboxylic groups of methotrexate molecules to activate their reactivity with primary amines functions.

3- Add 70 mg of sulfo-NHS ($2,05 \times 10^{-1}$ mmol*).*

Sulfo-NHS stabilizes the EDC activated carboxylic groups, optimizing the efficiency of the methotrexate-annexin conjugation.

4- Stir vigorously for 5 min.

5- Add 2 µL of *β*-mercaptoethanol.

β-mercaptoethanol neutralizes the excess of EDC and NHS preventing their interference in downstream reactions.

6- Immediately titrate the solution to a pH of 7.4.

7- Add the methotrexate-sulfo-NHS solution dropwise to a 10 mL solution of 1 mg/mL of annexin A5 ($2,8 \times 10^{-4}$ mmol).

8- Stir gently for 12 hours.

9- Centrifuge 10 min at 12 000 g.

10- Retain the supernatant and discard the pellet.

11- Dialyze against 2 L of PBS for 3 hours at 4°C.

This step removes the rest of the unbound methotrexate as well as other upstream contaminants such as β-mercaptoethanol.

12- Filter the supernatant with a 0.2 µm filter.

13- Flash freeze the filtered conjugate solution then store it in -80°C liquid nitrogen.

Optimized ANXA5-MTX conjugation protocol (3.2 mg of conjugate)

1- Dissolve 3 mg of methotrexate and 3 mg of EDC in 0.5 mL DMSO and protect the vial from light using foil.
2- Place the solution in a 50°C water bath for 30 min. *This step promotes the esterification of methotrexate by EDC.*
3- Cool the solution to room temperature and add it dropwise to a 2 mL PBS solution of 1.6 mg/mL of annexin A5 at a pH of 7.4. *Make sure the solution is continuously stirred to promote the dissolution of the chemical reagents and titrate the pH of the solution before proceeding to the next step.*
4- Stir gently for 30 min at room temperature. *At the end of this step, the solution should be clear and yellow.*
5- Dialyze the solution against 2L PBS pH 7.4 for 2 hours, change the media and redialyze for 2 hours. *This step allows for the discard of undesired chemical reagents (NHS, EDC, free MTX).*
6- Retrieve the solution and centrifuge it at 3000 g for 5 min. The final solution should be yellow (at least slightly) and clear. If protein precipitates persist, recentrifuge the sample as priorly said. *This step allows to eliminate the precipitated and cross-linked annexin A5 and the remaining free methotrexate*.
7- Filter the supernatant with a 0.2 µm filter.
8- Flash freeze the filtered conjugate solution then store it in -80°C liquid nitrogen.

Alamar Blue assay protocol

All steps from 1 to 8 are performed in sterile conditions.

1. Prepare 15 mL of 40 000 cells/mL of media.

2. Seed the central 60 wells (B2 – G11) of a 96 wells plate with 200 µL of the suspension containing 10 000 cells per well. Fill 3 wells with media without cells to establish the noise of the fluorescence. Fill the 30 outer wells of the plate with 200 µL of PBS to limit evaporation in the incubator.

3. Let the plate incubate for 24 hours.

4. When confluency has reached 70%, proceed to treatment: empty the wells with a multichannel pipette and refill them with 180 µL of fresh media.

5. Add 20 µL of the appropriate treatment samples to the wells. The untreated and blank controls will be supplemented with 20 µL of PBS.

6. Incubate cells for 48 hours.

7. Carefully empty the wells of the plate using a multichannel pipette and refill them with 200 µL of a priorly prepared solution of 10% Alamar Blue reagent in cell culture media.

8. Incubate cells with Alamar Blue for 2-4 hours to allow dye uptake and reduction.

9. Transfer the content of the plate to a black 96-well plate by using a multichannel pipette and by respecting the right order of wells.

10. Read the fluorescence of the plate at 530 nm excitation and 590 nm emission and subtract the blank fluorescence value.