Liposomal Curcuma as delivery system

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Abstract

Liposomes could enhance antitumor and pharmacological activities of Curcumin by improving pharmacokinetics and pharmacodynamics and reduce the dosage required for targeting tumor. Curcumin, the turmeric isolated natural phenolic compound, has shown a promising chemo-preventive and anticancer agent. Numerous studies have shown that curcumin delays the initiation and progression of non-small-cell lung carcinoma by affecting a wide range of molecular targets and cell signaling pathways. However, the poor oral bioavailability and low chemical stability of curcumin remain as major challenges in the utilization of this compound as a therapeutic agent. A special liposomal formula by MITO-Biomedical GmbH & CO. KG with notably high amounts of phosphatidylcholine (PC) could be a new approach for getting liposomes to function as an optimal delivery system of drugs. This project aims to improve Curcumas pharmacokinetic profile and show if Curcuma both has an tumoricidal effect and an inhibitory effect on the activation of STAT3.

Keywords: Drug delivery system, Liposomes, Curcuma

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Abbreviations

PC	Phosphatidylcholine
TAM	Tumor-associated macrophages
STAT3	Signal transducer and activator of transcription 3
CU-LIPO	Curcuma-loaded liposomes
WHO	World Health Organization
IV	Intravenously
PBS	Phosphate-buffered saline
EPR	Enhanced permeability and retention
GFP	Green fluorescent protein
qPCR	Quantitative Polymerase Chain Reaction
HPSEC	High Pressure Size Exclusion chromatography
CT	Threshold cycle
DLS	Dynamic Light Scatter
ATP	Adenosine Tri Phosphate
PBMC	Peripheral blood mononuclear cells
MDM	Human monocyte-derived macrophages
αCD163	Anti- CD163 antibodies

Aim of the study

This study aims to verify the effectiveness of high phosphatidylcholine (PC) liposomes with Curcuma by targeting Tumour-associated macrophages (TAMs). TAMs are known to be important in cancer-related immune suppression together with tumour infiltration by CD163pos are TAMs associated with a reduced chance survival in most human cancers. Curcuma has been reported to function as an anti-cancer drug and more specific shown to be have an inhibitory effect on STAT3, but both difficulties absorbing curcuma and targeting cells is a known problem. We therefore target the endocytic CD163 scavenger receptor, which is highly expressed on TAMs, by modification of the liposomes with curcuma with monoclonal anti- CD163 antibodies (αCD163)- CU-LIPO-αCD163. The special formula of liposomes therefore aims to both achieve a higher absorption in $CD163_{pos}$ cells and longer systemic circulation time thus decreasing the clearance of Curcuma and moreover targeting STAT3. Furthermore, an investigation of Curcumas direct tumoricidal effect will be studied on the hepatic cancer cell line AML12.

1. Introduction

1.1 Liposomes

The efficacy of the pharmaceutical formulation of ingredients in liposomes for different applications has become of interest recently. Especially in anti-cancer treatments are liposomes a hot topic. Liposomes are well-known drug delivery systems that have been successfully used for the delivery of several drugs and phytochemicals. Liposomes are constructed of a phospholipid vesicle consisting of one or more concentric lipid bilayers enclosing discrete aqueous spaces. The unique ability of liposomal systems to entrap both lipophilic and hydrophilic compounds enables a diverse range of drugs to be encapsulated by these vesicles coupled with their biocompatibility and biodegradability, low toxicity, high loading capacity, and controllable release kinetics, makes liposomes an attractive vehicle in the field of drug delivery. Hydrophobic molecules are inserted into the bilayer membrane, and hydrophilic molecules can be entrapped in the aqueous centre one or more phospholipid bilayers surrounding an aqueous inner space. Nevertheless, have conventional liposomal formulations not showed great success in vivo. This is due to liposomes tending to fuse of aggregate with each other resulting in immature release of liposomal payload over time (1). Besides, conventional liposomes underwent rapid systemic clearance *via* their uptake by the cells of mononuclear phagocyte system (2, 3). Several engineering strategies have been applied to improve the in vivo performance of liposomes. These strategies include either PEGylated liposomes the attachment of site-directed surface ligands, such as antibodies (immunoliposomes) (4), positive charge (cationic liposomes) (5), or a peptide (peptide-targeted liposomes) (6), or exploiting the inherent physiological conditions in the target tissue, such as elevated temperature or alteration in pH so as to produce stimuli-responsive liposomes such as thermosensitive liposomes (7) and pH-sensitive liposomes (8). Liposomes with a good quality of phospholipids, with high amounts of PC, do show a much greater bioavailability of active ingredients in targeted organs/cells, than in conventional formulations. PC is the main phospholipid of cellular membranes and the natural form of the vitamin-like nutrient choline. PC plays an important role in the digestion and absorption of lipophilic nutrients and exerts a protective barrier function in the gastrointestinal tract. PC promotes the formation and secretion of lipoproteins from the liver to the tissues and the transport of triglycerides, cholesterol, and phospholipids in the blood plasma. Membrane replacement therapy with PC intravenously or oral administration is to be used to enhance the function of cellular membranes. The specially engineered liposomes from MITO-Biomedical with a very high concentration of PC and liposome size not higher than 100 nm should promote optimal cellular uptake. This study gives the opportunity to scientifically proof this

liposomal formula and compare it to conventional delivery methods (9). Liposomal curcumin has a higher stability (in PBS), a reduced clearance, a longer half-life, a higher bioavailability and a lower toxicity on healthy cells compared to non-coated curcumin (10, 11). In addition, a higher uptake and a stronger tumour-suppressive effect of liposomal compared to free curcumin was confirmed in different cells (12).

1.2 Curcuma

1.2.1 General

Curcumin, a natural phenolic compound extracted from the rhizome of the plant *Curcuma longa*, which is a member of the curcuminoid family. The characteristic yellow color of the turmeric is due to the curcuminoids present in it, namely curcumin, demethoxycurcumin, bisdemethoxycurcumin, and cyclocurcumin. In addition, the curcuma plant consists of essential oils (3-7%), minerals (3-7%), proteins (6-8%), fats (5-10%), a water / moisture content of 6-13% and Carbohydrates (60-70%). The curcuminoids can be divided into three main components: curcumin (60-70%), demethoxy curcumin (20-27%) and bisdemethoxy curcumin (10-15%), in addition to some secondary metabolites (13). Curcuminoids are found in 3% to 5% of turmeric, and curcumin is the major bioactive constituent (14). Curcumin is of great interest to researchers because it has a wide variety of bioactivities, including its antioxidant, antimicrobial, anti-inflammatory, antitumor, antidiabetic, hypolipidemic, hepatoprotective, and neuroprotective effects (15, 16). Curcumin has been reported to prevent many diseases through modulating several signaling pathways, and the molecular bases of its anti-tumor bioactivities are imputed to the anti-proliferative, anti-inflammatory, pro-apoptotic, anti-angiogenesis and anti-metastasis effects (16). Although curcumin is a safe and promising phytochemical, it suffers from bioavailability problems that limit its therapeutic efficacy. Thus, various promising strategies allowed for the obtainment of multiple and effective varieties of curcumin formulations such as adjuvants, nanoparticles, analogues, micelle and phospholipid complexes, metal complexes, derivatives, and as in this project also liposomes (17).

1.2.2 Pharmacokinetics of Curcumin

Curcumin is also called diferuloylmethane or according to IUPAC nomenclature as (1E, 6E) -1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione and has the chemical formula $C_{21}H_{20}O_6$ (13). There are three main types of curcuminoids including 1,7-bis-4-hydroxy-3-methoxyphenyl-

hepta-1,6-diene-3,5- dione (Figure 1,1, ~77%), 1,4-hydroxy-3-methoxyphenyl 7,4-hydeoxyphenyl-hepta-1,6-diene-3,5-dione (Figure 1,2, ~17%) and 1,7-bis-4-hydeoxyphenyl-hepta-1,6- diene-3,5-dione (Figure 1,3, ~3%). The main commercial Curcumin is number 1 in Figure 1, which belongs to Biopharmaceutical Classification System Class IV. The molecular weight and melting point of Curcumin is 368.37 g/mol and 183°C, respectively. Curcumin extremely sensitive to light, while temperature has little influence on its stability even at 250°C. In solution, when the pH value is >5, Curcumin is unstable and its degradation rate significantly speeds up with increasing the pH value of the solution (18, 19).

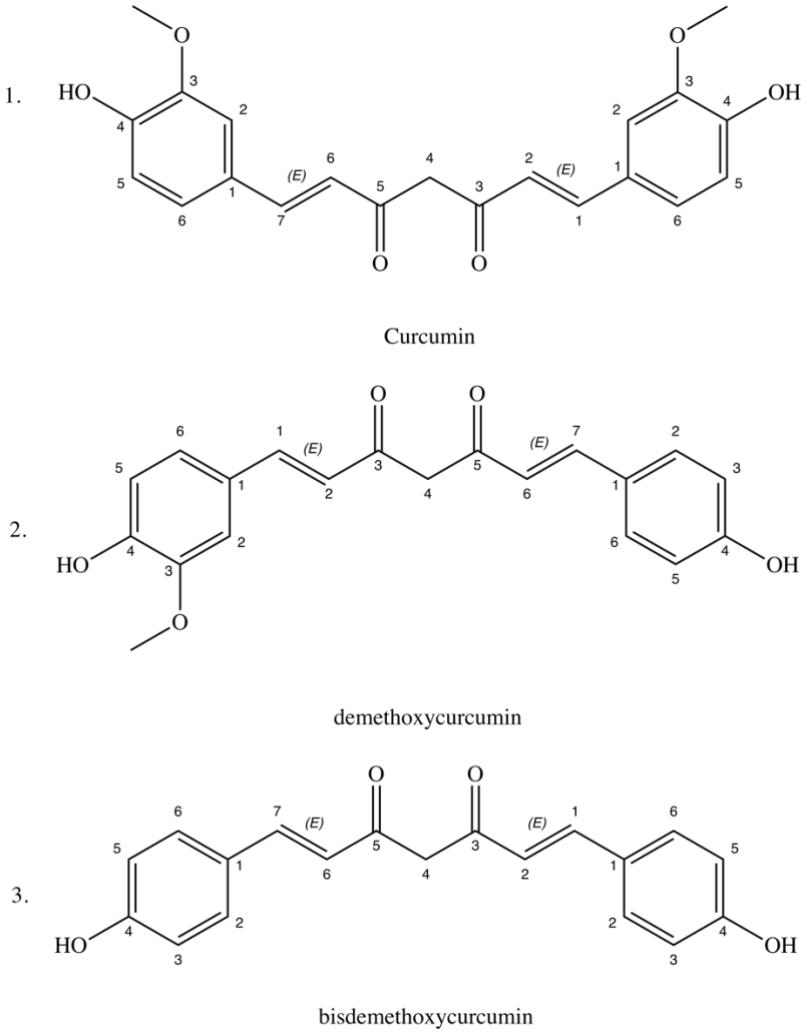

*Figure 1: Curcuminoid extract, **1** makes up 60–70% by weight, while **2** (20–27%) and **3** (10–15%) are more minor components. The major constituent of a curcuminoid extract, **1**, and the properties important for its consideration as a lead compound for therapeutic development are the focus of this review (20) .*

As can be seen from the structural formula, curcumin is a β-diketone, which consists of two ferulic acids linked via methylene. In addition to the β-diketone group and some carbon-carbon double

bonds, curcumin contains two benzene rings, each with a hydroxyl and methoxy group. Accordingly, curcumin is an amphiphilic molecule with lipophilic and hydrophilic properties. Curcumin is also a keto-enol tautomer (21). While the bis-keto form predominates at an acidic and neutral pH, the enol form predominates at an alkaline pH. Furthermore, curcumin has a low solubility in water, but an increased solubility in protic and aprotic polar solvents (22, 23). This low water solubility leads to a low absorption when administered orally (24). In addition, rapid metabolism and rapid excretion cause the low oral bioavailability of curcumin (25). In order for curcumin to be used in therapy, a certain therapeutic concentration in the blood and tissues must be achieved. For this purpose, the absorption, the distribution in the tissue and the plasma half-life must be improved, overall the bioavailability must be improved. One of these approaches is through liposomes as a drug delivery system. The toxicity of Curcumin has also been examined before use as a therapeutic agent. Various preclinical studies in rats, dogs and monkeys had shown that curcumin did not induce any significant toxicity (24). Furthermore, the World Health Organization (WHO) defined the ADI value (acceptable daily intake) as 0-3 mg / kg body weight. A usual therapeutic dose is 400 - 600 mg curcumin three times a day, which corresponds to about 60 g of the curcuma plant or 15 g curcuma powder (26). The only side effects observed was in a study, where subjects received 0.45 to 3.6 g/day curcumin for one to four months reported nausea and diarrhea and an increase in serum alkaline phosphatase and lactate dehydrogenase contents (27).

1.2.3 Pharmacodynamics of Curcumin

So far, many effects of curcumin have been published, including the antioxidative, anti-inflammatory, antibacterial and tumor suppressive effects (28). The focus of this work will be on the tumor suppressive effects of curcumin. Therefore, only these aspects are dealt with below.

1.2.3.1 Tumor-suppressiv effect

It has been shown that Curcumin can be used for treatment of cancers including lung, cervical, prostate, breast, osteosarcoma and liver cancers. In this experiment we focus on hepatic cancer. Numerous studies have indicated that anticancer properties of Curcumin are related to enzymes such as COX-2, AMPK, MMPs, NADPH and LOX; transcription factors such as NF-κB, AP-1, β-catenin and STAT-3 and protein kinases and growth factors such as MAPK, AKT, JAK, VEGF, ERK, PKA and Bcl-2. Liposomes have been used in the delivery of anticancer drugs and are able to alter the biodistribution and clearance of drug molecules (29). Intravenously (iv) administered liposomes are

taken up by the reticuloendothelial system (RES) after entering the body. Liposomal drugs mainly accumulate in the liver, spleen, lung, bone marrow and primary in phagocytic cells like macrophages so as to improve the therapeutic index of drugs and reduce their side effects. With the broad application of liposomes, more novel liposomes such as long-circulating liposomes and ligand-modified liposomes have been designed to prolong action time of drugs in blood and target different cancers. Thus, combination of Curcumin and liposomes should enhance the stability, bioavailability, targeting property and anticancer efficacy of Curcumin. It has been reported that Curcumin induced apoptosis of liver cancer cells mainly happens through regulation of apoptosis-related proteins including the Bcl-xl and the Bcl-xs. Control cytochrome C and ROS release and by adjusting the cyclin and induce cell cycle arrest including caspase-3 and caspase-8 pathways. Besides, Curcumin can lead to mitochondrial and nuclear DNA damage in liver cancer cells, especially its mitochondrial DNA (30). It has been shown by Ren et al that Curcumin inhibits proliferation, invasion, and metastasis in human liver cancer cells (HEPG2). It caused the cells to remain in the DNA S phase, promoted apoptosis, and significantly reduced intracellular heat shock protein 70 (HSP70) and the toll-like receptor 4 (TLR4) levels of HepG2TT cells (thermal tolerance). Following the removal of curcumin, extracellular HSP70 increased again (31). Another study reported that Curcumin could inhibit growth and angiogenesis of tumor and significantly reduce expression of COX-2 and VEGF in HepG2 liver cancer tissues (30).

1.3 Tumor-associated macrophages

Macrophages are exceptionally diverse in their functions reflecting the different origins, local environment and responses to challenges (32). Consideration of macrophage function in immunity let to the proposal of two classes of macrophages: 1) the activated macrophages responding to IFN-γ, TNF-α and toll-like receptor 4 (TLR4) activation capable of killing pathogens through mechanisms such as iNOS; 2) Alternatively activated macrophages responding to IL-4 and IL-13 involved in anti-parasitic immunity and in asthma. The original in vitro characterizations were extended to in vivo models and called M1 (activated) and M2 (alternatively activated). These descriptions were captured to suggest that TAMs could be either tumor killing (M1) or tumor promoting (M2) (32). TAMS have been shown to be of major importance in the establishment of tumor environment that is promoting tumor growth, angiogenesis, resistance to anti-tumor immunity and chemotherapy and lastly metastasis (33). Results display that high TAM-infiltration is associated with poor outcome in the majority of human malignancies (34). In general, the TAM phenotype is anti-inflammatory (M2-like)

in contrast to pro-inflammatory and potentially tumoricidal macrophages (M1-like) (32). It has been shown that a pro-inflammatory stimulus can reprogram TAMs from a tumor-promoting M2-like phenotype, towards tumoricidal M1-like cells (33). TAMs are thus proposed as highly attractive targets for novel immunomodulatory anti-cancer therapy. As Andersen et al. (33) proposes that a more effective strategy of immunomodulation is to reprogram TAMs from tumor promoting (M2-like) to a tumoricidal (M1-like) phenotype by inhibition of the transcription factor signal transducer and activator of transcription 3 (STAT3) within TAMs. STAT3 is thus targeted due to its role in in several human cancers (e.g. colorectal, gastric, hepatocellular, and ovarian carcinomas) which was associated with immune suppression, as well as tumor cell proliferation, survival, and invasion(33) . Papers show that STAT3 in CD163pos TAMs within human tumors, has anti-tumor effects of ERK5 inhibition were mediated by inhibition of STAT3 (35). The hemoglobin–haptoglobin scavenger receptor CD163 is among the most well-described markers of M2-polarized macrophages, and especially TAMs (32, 33). Infiltration by CD163pos TAMs is also associated with poor outcome in several human cancers and CD163 and the anti-inflammatory cytokine IL-10 are both markers of M2-polarized macrophages (including TAMs), and may be upregulated in vitro by tumor cell supernatant or IL-10 itself via activation of STAT3 (36). CD163 is an endocytic receptor with high expression on the TAMs, it could be the gateway for delivery of anti-cancer therapeutics. Recently, Andersen et Al. developed an anti- body/liposome-based system that allows effective targeting of compounds to CD163-expressing cells. As mentioned before are liposomes attractive carriers for delivery of anti-cancer drugs to tumor tissues, due to increased circulation time and passive accumulation of liposomes in tumors by the enhanced permeability and retention (EPR) effect. As tumor blood vessels are leaky and lymphatic vessels less functional this leads to passive accumulation of liposomes in vivo (37). Thus, a CD163-targeted STAT3 inhibitor may be a novel effective and specific anti-cancer drug, as it may activate anti-tumor immunity by reprogramming TAMs. Furthermore, Etzerodt et Al. showed that removal of the specific CD163+ TAMs results in enhancement of immunoinfiltration, which in turn results in an anti-tumor effect (38). We aim to develop another liposome-based STAT3-inhibitory drug targeting human CD163pos cells (including TAMs) besides the one Andersen et Al. has made with Corosolic acid and instead using Curcuma.

1.4 Introduction to methods

To detect and quantify the efficacy of the liposomes with the encapsulated compound Curcumin ATPlite Assay was used. Flow cytometry and confocal microscopy was performed to staining of all

antibodies would be done. Furthermore, a gene expression analysis by quantitative polymerase chain reaction (qPCR) will be performed. Liposome size assessment was performed using dynamic light scatter (DLS). Concentrations of lipid and Curcuma will be measured using high-pressure size-exclusion chromatography (HPSEC).

1.4.1 ATPlite

ATPlite™ is an Adenosine TriPhosphate (ATP) monitoring system based on firefly (Photinus pyralis) luciferase. This luminescence assay is the alternative to colorimetric, fluorometric and radioisotopic assays for the quantitative evaluation of proliferation and cytotoxicity of cultured mammalian cells. ATP monitoring can be used to assess the cytocidal, cytostatic and proliferative effects of a wide range of drugs, biological response modifiers and biological compounds. ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. When cells lose membrane integrity, they lose the ability to synthesize ATP and endogenous ATPases rapidly deplete any remaining ATP from the cytoplasm. The ATPlite assay system is based on the production of light caused by the reaction of ATP with added luciferase and d-luciferin. This is illustrated in the following reaction scheme:

$$\boldsymbol{d-Luciferin + ATP + O_2 \rightarrow Lucierferacse \rightarrow Oxyluciferin + AMP + PPi + CO_2 + Light}$$

The emitted light is proportional to the ATP concentration within certain limits. A limitation associated with common luciferase assay technology is the short half-life of the light emission. This flash-type signal requires luminometers with reagent injectors to measure the quick reaction. ATPlite is a mixture of several substances that extends the signal half-life to over 5 hours. A problem with some ATP assay kits that are currently on the market is that the lysing solutions that release the ATP do not irreversibly inactivate endogenous ATP degrading enzymes (ATPases). Also, some lysing solutions contain chaotropic agents like TCA, which 5 ATPLite have a negative effect on the luciferase activity. The ATPlite kit overcomes these problems by raising the pH of the cell culture medium through the addition of the mammalian cell lysis solution. The lysis solution inactivates the endogenous ATPases. The subsequent addition of the substrate solution (Luciferase/Luciferin) lowers the pH to a suitable level so that the reaction can occur (39). The ATP detection reagent contains detergent to lyse the cells, ATPase inhibitors to stabilize the ATP that is released from the lysed cells, luciferin as a substrate, and the stable form of luciferase to catalyse the reaction that generates photons of light.

1.4.2 Flow cytometry

Flow cytometry is a powerful tool for interrogating the phenotype and characteristics of cells. It is based upon the light-scattering properties of the cells being analyzed and these include fluorescence emissions. All forms of cytometry depend on the basic laws of physics, including those of fluidics, optics, and electronics. Flow cytometry is a system for sensing cells or particles as they move in a liquid stream through a laser (light amplification by stimulated emission of radiation)/light beam past a sensing area. The relative light-scattering and color-discriminated fluorescence of the microscopic particles is then measured (33). Analysis and differentiation of the cells is based on size, granularity, and whether the cell is carrying fluorescent molecules in the form of either antibodies or dyes. As the cell passes through the laser beam, light is scattered in all directions, and the light scattered in the forward direction at low angles (0.5–10°) from the axis is proportional to the square of the radius of a sphere and so to the size of the cell or particle. Light may enter the cell and be reflected and refracted by the nucleus and other contents of the cell; thus, the 90° light (right-angled, side) scatter may be considered proportional to the granularity of the cell. The cells may be labeled with fluorochrome-linked antibodies or stained with fluorescent membrane, cytoplasmic, or nuclear dyes. Thus, differentiation of cell types, the presence of membrane receptors and antigens, membrane potential, pH, enzyme activity, and DNA content may be facilitated (40, 41).

1.4.3 Confocal microscopy

Similar to the widefield microscope, the confocal microscope uses fluorescence optics. Instead of illuminating the whole sample at once, laser light is focused onto a defined spot at a specific depth within the sample. The Laser (energy source) scans the specimen across and down to build the image. This leads to the emission of fluorescent light at exactly this point. A pinhole in front of the detector so light from other focal planes are blocked. It scans each focal plane at a time so that you can get clear images where two proteins contact. Thus allowing only the fluorescence signals from the illuminated spot to enter the light detector. This technique is always used to confirm what is seen with green fluorescent protein (GFP) (33, 42).

1.4.4 Dynamic Light Scatter

Dynamic light scattering is a well-established, standardized technique for particle size analysis in the nanometer range and has been used for about 40 years. DLS provides information on the mean particle

size as well as on particle size distribution. It covers a broad size range from the lower nanometer range up to several micrometers (33). Only low sample volumes are required and the sample can be re-used after the measurement. Dynamic light scattering (DLS) is based on the Brownian motion of dispersed particles. When particles are dispersed in a liquid they move randomly in all directions. The principle of Brownian motion is that particles are constantly colliding with solvent molecules. These collisions cause a certain amount of energy to be transferred, which induces particle movement. The energy transfer is more or less constant and therefore has a greater effect on smaller particles. As a result, smaller particles are moving at higher speeds than larger particles. If you know all other parameters which have an influence on particle movement, you can determine the hydrodynamic diameter by measuring the speed of the particles (43).

1.4.5 Quantitative Polymerase Chain Reaction (qPCR)

qPCR has entered widespread use with the increasing availability of real-time PCR. By the incorporation of fluorescent dyes in the reaction mixture, increases in amplification products can be monitored throughout the reaction, enabling measurements to be taken in the exponential phase of the reaction, before the reaction plateau. In qPCR, DNA amplification is monitored at each cycle of PCR. When the DNA is in the log linear phase of amplification, the amount of fluorescence increases above the background. The point at which the fluorescence becomes measurable is called the threshold cycle (CT) or crossing point. By using multiple dilutions of a known amount of standard DNA, a standard curve can be generated of log concentration against CT. The amount of DNA or cDNA in an unknown sample can then be calculated from its CT value. qPCR is a collection of methods for measuring amounts of specific template DNA sequences (44).

1.4.6 HPSEC

High Pressure Size Exclusion chromatography (HPSEC) is increasingly used to evaluate molecular sizes of humic substances from different sources. Normally a HPLC system with an auto sampler and a photodiode array detector to separate and detect natural organic matter of different molecular weight (or size) range in water samples. The data acquisition and absorbance chromatograms are controlled by a software. HPSEC is confirmed to represent a highly precise method to evaluate the relative molecular-size distribution of dissolved humic substances (45).

2. Methods

To show that the activation of STAT3 by phosphorylation will be inhibited by CU-LIPO-aCD163. For this to be verified CD163+ cells and CD163- cells will be made from a monocyte/macrophage lineage. To show the level of liposomal compound that fuses into the cell compared to non-liposomal encapsulated compounds, an AML12 (#ATCC CRL2253) which is an alpha mouse liver cell line, will be used. It is of interest to show if the Curcuma generates apoptosis of the cells. Exposure was performed with 50 µM (50mg/ml) in a serial 2-fold dilution for up to 20 hours and a control with no compound was used. 2 times 96 samples will be generated (96x Curcumin-liposomal encapsulated, 96x Curcumin-non-liposomal. Notably, culturing of AML12 was done before COVID-19 and lockdown of laboratory facilities.

2.1 Culturing, exposure and sample preparation of AML12

The AML12 cell line will be established from hepatocytes from a mouse (CD1 strain, line MT42) transgenic for human TGF alpha. The cells were grown in T75 flasks for several passages and maintained by splitting the cells 2 times a week and renewing the medium every 7th day. Flasks will be checked before passaging and should have 80-90% confluency which will typically have 2 to 3 million cells in a flask. Splitting of cells was done with Phosphate-buffered saline (PBS), which was pre-heated to 37 °C before use. The old medium was removed by the use of a suction pump and the cells were washed with PBS. Trypsin solution was added to the cells to detach them from the surface of the flask/dishes, and the flask/dishes were tilted for the distribution of the liquid. The flask/dishes were incubated at 37 °C and 5% CO_2, and the cells were investigated under the microscope after trypsinization. The cells were then stimulated with 50 *µM (50mg/ml)* for up to 20 hours (46). The media for AML 12 cells was composed of DMEM: F12 Medium (ATCC 30-2006). To make the complete growth medium, the following component will be added to the 500 mL base medium, 10% fetal bovine serum (FBS; ATCC 30-2020), 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone. This medium is formulated for use with a 5% CO_2 in air atmosphere.

2.2 Production of high PC liposomes

Liposomes will be made at MITO-biomedical and differs from other liposomes in their high amount of PC. The liposomes will after production be subsequently remote loaded with Curcuma. For remote loading of the produced liposomes, a solution of Curcuma (MITO- biomedical, Germany) in PBS with 1% DMSO will be made. This will be mixed with the liposomes and suspension in a buffer with

35 mM citric acid and 0.85 mM Curcuma and incubated for 60 min at 65 °C in a shaker incubator. After this incubation, the solution containing liposomes will be transferred to micro-centrifuge tubes, and centrifuged for 5 min at 400g to remove excess Curcuma (33). To target Curcuma-loaded liposomes (CU-LIPO) to the CD163 scavenger receptor, CU-LIPO will be modified by insertion of a lipidated anti-human CD163 antibody into the CU-LIPO (αCD163, clone KN2/NRY, humanized IgG4. KN2/NRY insertion will be done by PEGylation of the antibodies using 4-nitrophenol-coupled PEG3400 that will be also coupled with the phospholipid DSPE to facilitate passive insertion into the liposome lipid bilayer. After this modification, CU-LIPO without antibody as well as KN2/NRY-modified CU-LIPO (CU-LIPO-αCD163) will dialyzed twice (300 kDa cut off, Float-A-Lyzer, Spectrum Labs, Breda, Netherlands) against isotonic PBS, and will then be sterile-filtered (0.2 μm) and stored at 4 °C (33).

2.3 Purification of peripheral blood mononuclear cells (PBMCs)

Human PBMCs will be purified from buffy coats obtained from the blood bank. The PBMC fraction will be purified by density-gradient centrifugation (400g, 30 min) on a Histoqaque-1077 gradient (Sigma-Aldrich) according to the manufacturer's instructions. After purification and wash in PBS/2% fetal calf serum (FCS), PBMCs will be frozen at − 80 °C in RPMI-1640 medium with 20% FCS and 100 U/100 μg/mL penicillin/ streptomycin and 10% DMSO (Sigma-Aldrich), or will be used in experiments immediately. PBMCs will be used to purify CD14pos monocytes for culture of human monocyte-derived macrophages, the cells will not be frozen before this purification step (33). An alternative is the use of cells that express recombinant CD163, which is easier to use in the early stages of the experiment then PBMCs (47).

2.4 Culture of human monocyte-derived macrophages (MDMs)

Human MDMs will be cultured from monocytes purified from PBMCs by EasySepTM human CD14-positive selection kit, according to the manufacturer's instructions. After isolation and wash, monocytes will be cultured for 5–6 days in RPMI-1640 medium with 10% FCS, 100 U/100 μg/mL pen/strep, 10 ng/ mL M-CSF, and 1 ng/mL GM-CSF to induce differentiation into MDMs. For CD163-targeting experiments, cells will then be stimulated with IL-10 (20 ng/mL, PeproTech) for 2–3 days to induce CD163 expression in MDMs. For harvesting adherent MDMs, cells will be first loosened by 10–15 min incubation with PBS/0.5% bovine serum albumin (BSA)/5 mM EDTA/4

mg/mL Lidocaine (Sigma-Aldrich). Cells will then be detached by pipetting and/or by scraping with a cell scraper (33).

2.5 Gene expression analysis by quantitative polymerase chain reaction (qPCR)

Cultured human MDMs will be dissolved in RLT buffer and RNA will be purified using the RNeasy mini kit (Qiagen, Hilden, Germany). Purified RNA will be reverse-transcribed to cDNA in a reaction with 2.5 U/μL MulV reverse transcriptase enzyme (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA), 1.0 U/μL RNase inhibitors (Applied Biosystems), 1.0 mM dNTP mix (VWR International, Radnor, PA), 2.5 μM Oligo(dT) (DNA Technology, Risskov, Denmark), 1 × PCR buffer and 6.25 mM MgCl2 (from Applied Biosystems). RNA input will be 100 ng in a 20 μL reaction (RNA concentrations will be measured using a NanoDrop 2000 spectrophotometer, Thermo Fisher Scientific). Quantitative PCR will be performed using a LightCycler 480 instrument (Roche, Basel, Switzerland) in a 10 μL reaction with 1 μL cDNA, primers, and 480 SYBR Green I Master mix (Roche) according to the manufacturer's instructions. Samples will be run in duplicates. A standard curve will be included in all runs, and will be used to calculate relative concentrations of target mRNA. All qPCR results on human MDMs will be normalized to the levels of GAPDH (housekeeping gene) (33).

2.6 Measurement of pro-inflammatory cytokines within culture medium/supernatant

Using electro-chemo-luminescence-based multiplex sandwich immunoassays the measured levels of IFNγ, TNFα, IL-2, and IL-12 according to the manufacturer's instructions (Human Pro-inflammatory panel, Meso Scale Discovery, Rockville, MD). All medium samples will be diluted 1:2 in assay dilution buffer (33).

3. Conclusion and further perspectives

As the aim of this project both was to inhibit activation of STAT3 by CU-LIPO-αCD163 and to show that the tumoricidal effect of Curcuma on cancer cells is higher when encapsulated in the special formula of liposomes provided by MITO-biomedical. The aims of the study could not be researched due to restricted access to laboratory facilities due to COVID-19. This paper is as mentioned therefore only theoretical. Regarding CU-LIPO-αCD163 inhibition of STAT3 it would be expected to see an inhibition of STAT3 mostly within CD163+ due to reports of Curcumas inhibitory effect on STAT3.

As Andersen et al. stated could this targeting increase the effects and decrease side effects, of future STAT3-inhibitiory anti-cancer therapy (33). Regarding the tumoricidal effect of liposomal Curcuma on AML12 cells, would it be expected to see a higher concentration of Curcuma inside the cancer cells incubated with liposomal Curcuma due to the expectations that encapsulation with liposomes enhances bioavailability of Curcuma. Looking forward this experiment would eventually be divided into two studies, whereas the liposomal function and Curcumas tumoricidal role firstly would be inspected before testing Curcumas effect against TAMs. If the experiments indicate that both the liposomal delivery of Curcuma is enhanced and that it has a tumoricidal effect an ultimately has an inhibitory effect on the activation of STAT3, then a in vivo study could be conducted. Furthermore, a study where the liposomes from MITO-biomedical could be compared to the conventional liposome would enlighten if this special formula of liposome should be favoured when making liposomal studies.

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