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AN APPROACH FOR MEASURING THE RELEASE OF AN ENDOPHYTIC BIOACTIVE OXYLIPIN FROM SOLID LIPID NANOPARTICLES

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ABSTRACT

Endophytes are considered a promising source of new bioactive secondary metabolites and are found in almost all plants. Lipid nanoparticles are of interest as a carrier for these bioactive metabolites. It is necessary to fully characterize the drug retention and release properties of these nanoparticles loaded with these bioactive metabolites. Ion exchange column technique was used to study drug transfer from such carriers to lipophilic acceptor particles. An endophytic fungus was isolated from the medicinal plant *Bidens bipinnata*. The transfer of the bioactive metabolite from the negatively donor lipid nanoparticles, which were prepared from trimyristin, to the neutral acceptor unilamellar vesicles was examined after separation between donor and acceptor on an ion exchange column. The ethyl acetate extract of the fungal isolate exhibits antibacterial activity as well as cytotoxic and antiproliferative effects. Activity-guided chromatographic fractionation resulted in the isolation of the active metabolite which was identified as a new bioactive oxylipin. The transfer of this bioactive oxylipin was rapid but it stopped at a concentration less than the expected value of equal distribution. The medicinal plant *Bidens bipinnata* was chosen for the search of bioactive secondary metabolites due to its anti-inflammatory and antifungal effects. The low

equilibrium values might be attributed to the localization of the oxylipin at the interface of the acceptor unilamellar vesicles**.** Ion exchange column is a suitable technique to study the transfer of the bioactive metabolite oxylipin to acceptor unilamellar vesicles.

KEYWORDS: Ion exchange column, endophytic metabolite, drug transfer, unilamellar vesicles.

INTRODUCTION

Over the years, drug discovery has focused on microbial sources from which nearly 80% of the world`s antibiotics have been discovered. These sources have almost entirely been from soils collected from around the world, but new microbial origins need to be examined for the production of useful bioactive compounds. [1] Although it appears that the pharmaceutical industry has completely exhausted the hidden treasures of the microbial world to find solutions for the infectious diseases of the last century, a report by the American Academy of Microbiology estimates that less than 5% of fungal species are currently known and only 1% of these microbes have been cultured and characterized. Thus challenges to find novel microbes remain. Endophytes have been identified as a promising source of new pharmacologically active secondary metabolites that might be suitable for medicinal or agrochemical applications.

Bidens bipinnata is an annual weed distributed widely in the tropical and subtropical regions of the world. It is well-known as hairy beggar ticks or Spanish needles and is reported to be a weed of 31 crops in more than 40 countries. [2] It is an herbaceous plant widely distributed in Africa, America, China, and Japan that is used in traditional medicines for treatment of inflammation and various diseases, including hepatitis and diabetes. Furthermore, the ethanolic crude extract from the roots of *B. bipinnata* contains polyacetylenes and flavonoids that exert in vitro antimalarial activity against *Plasmodium falciparum.*[3]

Lipid nanoemulsions, which were introduced for the purpose of parenteral nutrition, are usually composed of fatty vegetable oils (e.g. soybean oil) or medium chain triglycerides as the lipid phase. During recent years it has been recognized that these systems may also be used as carriers for lipophilic drugs and several formulations have been commercialized. [4, 7] Advantages of nanoemulsions include toxicological safety and a high content of the lipid phase as well as the possibility of large-scale production by high-pressure homogenization. The possibility of controlled drug release from these lipid nanoemulsions is limited due to the

liquid state of the carrier. For most drugs, a rapid release of the drug has to be expected. [8, 11] The use of solid lipids instead of liquid oils is a very attractive approach to achieve sustained drug release from nanoparticulate lipid carriers, because drug mobility in a solid lipid should be considerably lower compared with liquid oil. Although a decrease in drug mobility has indeed been observed in lipid dispersions containing solid instead of liquid triglycerides. [12] this does not necessarily lead to slow release of drugs. [13] In order to rationally design lipid nanoparticles as colloidal carrier drug delivery systems and to obtain information on their potential in-vivo performance, it is thus necessary to fully characterize their drug retention and release properties. For this purpose, effective in-vitro assays have to be established.

Many methods have been described to investigate the in vitro drug release from colloidal drug delivery systems such as sample and separate methods [14-16], dialysis based assays [6, 17, 18], continuous-flow methods [19, 20] and in-situ techniques. [11] Not all of them appear suitable to obtain undistorted information on the release of lipophilic drugs into a large volume of release medium.[14, 20] As a closer approach to the in-vivo situation, the release medium can be supplemented with lipophilic particles like liposomes or emulsion droplets and the drug transfer into these particles be studied. [13, 21, 22] The ion-exchange mini-column model is an in vitro system for measuring the transfer of lipophilic drug molecules from the colloidal carrier systems to model membranes mimicking other membranous binding places in the body. This ion-exchange mini-column retains negatively charged donor particles, allows only the neutral acceptor particles to be eluted. [23] Neutral liposomes (mostly PC-liposomes) are used as acceptor medium at excess in relation to the donor particles. [23] In this study we present an approach to formulate bioactive metabolites isolated from an endophyte of the medicinal plant *B. bipinnata* into solid lipid nanoparticles and measure their in vitro release using the ion exchange column technique.

MATERIALS AND METHODS

Materials

The triglyceride trimyristin (D114, Dynasan 114) was a gift of Condea Chemie (D-Witten), sodium glycocholate (SGC), cholesterol, Trizma 7.4 pre-set crystals, sucrose, and sodium azide were from Sigma-Aldrich (D-Steinheim), egg phosphatidyl choline (EPC) and Lipoid S75 (S75) were obtained from Lipoid GmbH (D- Ludwigshafen), glycerol from Solvay GmbH (D-Rheinberg), thiomersal from Caesar and Loretz (D-Hilden), tris was from Carl Roth GmbH (D-Karlsruhe), Diethylaminoethyl (DEAE) Sepharose CL-6B was from

Amersham Biosciences AB (S-Uppsala), acetonitrile, ethanol, ethyl acetate, hexane and chloroform all from VWR International (D-Darmstadt), tetrahydrofurane (THF) was from Fisher Scientific (D-Nidderau), and sodium chloride from AppliChem GmbH (D-Darmstadt). Purified water was prepared by filtration and deionization/reverse osmosis (Milli RX 20, Millipore, D-Schwalbach).

Strain isolation and taxonomic classification

Samples of *B. bipinnata* were collected near Cairo, Egypt. After surface sterilization of the fresh, healthy, aerial plant parts an endophytic fungal strain was isolated. The strain was cultivated in four different culture media a malt extract (M4), a caseine–flesh peptone (M5), a cornsteep (M25) and a dextrose–yeast (M26) medium both as a shaken and as a stationary culture and was then subjected to antimicrobial activity screening. Results of the agar diffusion assay performed showed moderate antibacterial activity against *Bacillus subtilis.*

The strain was identified as *Khuskia oryzea* by ITS sequence comparison. Literature data showed that the only known metabolite of this rarely investigated strain was the antifungal agent griseofulvin. [24]

Endophyte fermentation, extraction and isolation

Large scale fermentation (60 L) in medium M25, where it showed the highest antimicrobial activity was carried out twice. Total extraction of culture broth and mycelium with ethyl acetate yielding 40 g of dried crude extract after solvent evaporation was carried out. Chromatographic fractionation of the extract was performed on silica gel using a solvent system of hexane: ethyl acetate starting with a proportion of 9:1 and then gradually increasing the proportion of ethyl acetate till final elution with 100% ethyl acetate. After combining the similar fractions three main fractions were obtained. The fraction Fa was found to contain the bioactive oxylipin 8-oxo-(9*E*, 11*E*)-octadecadienoic acid based on HPLC UV/MS measurements. It was evaporated to dryness giving a residue of 1 g which was formulated into solid lipid nanoparticles.

Preparation of trimyristin donor lipid nanoparticles containing the oxylipin

The dispersions were prepared from 5 % (w/w) trimyristin stabilized with 1.8 % (w/w) Lipoid S75 and 0.45 % (w/w) sodium glycocholate (SGC) in an aqueous phase containing 2.25 % glycerol for isotonization and 0.01 % thiomersal for preservation. The preparation was done by high-pressure melt homogenization using a Microfluidizer M-110S (Microfluidics, US-Newton). [25-28] S75 and SGC were dispersed / dissolved in the aqueous

phase by magnetic stirring overnight. The matrix lipid and the surfactant-containing aqueous phase were heated to 70 °C. The fraction Fa containing the bioactive oxylipin was dissolved in the molten lipid. The aqueous phase was poured to the molten lipid and the mixture was pre-homogenized for one minute (Ultra-Turrax T8, IKA Labortechnic, Germany). This crude emulsion was transferred to the warm (70°C) high-pressure homogenizer and treated for 5 min at 500 bar. The resulting hot colloidal emulsion was allowed to cool to room temperature. Under these conditions the matrix lipid remains in its liquid state due to supercooling. [29]

Preparation of the acceptor unilamellar vesicles

Acceptor unilamellar vesicles were prepared using EPC and cholesterol in a molar ratio of 8:2. [21, 30] EPC stock solution was prepared by dissolving 1.52 g in 20 ml chloroform and cholesterol stock solution was also prepared by dissolving 0.194 g in 20 ml chloroform. The two lipids (1 ml of each stock lipid solution) were mixed in a small bottom flask and dried to thin film under vacuum 200 mbar for 2 hours and then at 30 mbar for 1 hour (Büchi Rotavapor R-114, D-Essen). The resulting thin film was hydrated for 10 minutes with 1 ml tris buffer saline (10 mM tris, 140 mM NaCl, pH 7.4) under vortexing. The resulting lipid suspension was extruded through 200 nm membrane followed by extrusion through 100 nm polycarbonate membranes using Liposofast extruder (Avestin Europe GmbH, D-Mannheim).

Article size and zeta potential analysis

Particle sizes of the donor lipid nanoparticles with and without the bioactive oxylipin were measured by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK-Worcestershire). The dispersions were diluted with filtered demineralized water and measured at 25°C at a scattering angle of 173°. The results of three consecutive measurements of 5 min duration performed after 5 minutes of equilibration were averaged. The results are given as the z-average diameter and the polydispersity index (PDI, measure for the relative width of the particle size distribution). Zeta potential of the donor and acceptor particles was measured after diluting the samples with 10 mM tris buffer using the same Malvern Zetasizer Nano series (Malvern Instruments Ltd., UK-Worcestershire). The results of three consecutive measurements each consists of 20 runs were averaged.

Preparation of the ion exchange column

A total of 50 ml of DEAE-Sepharose CL-6B was washed twice with a 3-fold excess of tris

buffer saline (10 mM Tris, 140 mM NaCl, pH 7.4). After each washing the tris buffer was carefully decanted off and finally the gel was washed with a 3-fold excess of sucrose buffer (290 mM sucrose, 10 mM Trizma 7.4 pre-set crystals, 0.02% sodium azide, pH 7.4) and then diluted 1:1 (v/v) with sucrose buffer, which was also used for the elution of the columns. [21] The column length is 5 cm with an inner diameter of 0.5 cm. Some glass wool was placed at the bottom of the columns. About 1 ml ion exchange suspension (DEAE-Sepharose) was filled in the column and the column was eluted with 2 ml sucrose buffer for packing and the eluate was discarded. The columns were lipid saturated (to reduce non-specific adsorption and improve recovery of acceptor particles) by applying 20 µl of the acceptor and eluting with 1.5 ml sucrose buffer. [23] This eluate was also discarded. In all experiments, the elution of the columns was done using 1.5 ml sucrose buffer.

Oxylipin transfer to the acceptor unilamellar vesicles

The transfer experiments from the crystalline donor lipid nanoparticles to the acceptor unilamellar vesicles were carried out with molar ratios 1:25 and 1:100. Different amount of the donor solid lipid nanoparticles was added to Eppendorf tubes containing different amount of the acceptor unilamellar vesicles and sucrose buffer. The Eppendorf tubes were incubated for the intended times at 37°C. At appropriate time intervals 200 µl of the incubation mixture was placed on the ion exchange columns. The eluate was dissolved with a mixture of acetonitrile-tetrahydrofurane 20:80 (v/v) and the UV absorbance was measured at 230 nm.

Transfer kinetics

The transfer curves of the percental transferred amount of oxylipin to the acceptor unilamellar vesicles were exponentially fitted using Microcal Origin 6.0 software (OriginLab Corporation, US-Northampton) and the exponential function:

$$A_{acc} = A_{final} - A \times e^{-k \times t} \qquad [1]$$

A_{acc} is the percental amount of drug transferred to the acceptor particles at time t, A_{final} is the final percental transferred amount of drug and marks the height of the plateau, A is a pre-exponential coefficient and k is the rate constant of the transfer. The equilibrium time was determined by calculating the time required to reach 99% of the equilibrium amount.

RESULTS

Through activity-guided chromatographic fractionation the isolation of the fraction containing the active metabolites previously identified by different spectroscopic techniques

as a new bioactive oxylipin called 8-oxo-(9*E*, 11*E*)-octadecadienoic acid was achieved fig 1. In the assay performed for the compound for examination of its cytostatic activity, it was found to be of cytostatic effect against HUVEC and K-562 cancer cell lines. [31] In the antibacterial assay performed against *B. subtilis* moderate antibacterial activity was observed for it (inhibition zone 22 mm at a conc. of 250 µg mL^{-1}).

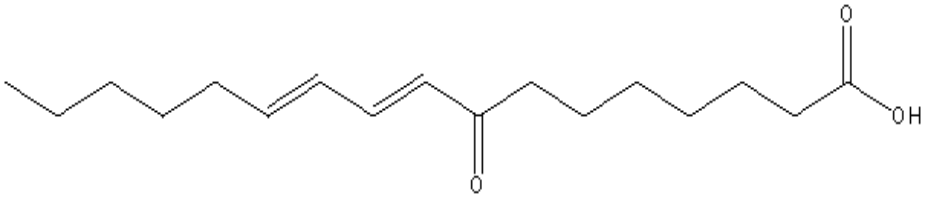

Figure 1. Chemical structure of 8-oxo-(9*E*, 11*E*)-octadecadienoic acid.

Particle size and zeta potential

Table 1 shows the particle size of the nanoparticles stored at room and refrigerator temperature. It could be observed that the nanoparticles stored at refrigerator temperature have a higher particle size because the recrystallization of the colloidally dispersed trimyristin is accompanied by an increase in the particle size and surface area which is due to the formation of platelet-like colloidal crystals with surface being structured by kinks, edges and steps. [32] The acceptor vesicles were prepared from egg phosphatidyl choline (EPC) and cholesterol with a molar ratio of 8:2 for EPC/cholesterol. EPC and cholesterol were chosen to mimic the cell membranes and other lipid compartments in the body and to obtain acceptor vesicles with neutral charge to be used on the ion exchange column. [21, 23, 30] As expected, particle size of the acceptor unilamellar vesilces that were extruded through a 100 nm membrane filter was in the nanometer size range. Negatively charged donors were, however, important for the nanoparticles to be suitable for the transfer experiments with the column technique. As seen from table 1, zeta potential of the donor nanoparticles was high and this could be attributed to the presence of S75. [33] In addition to the anionic emulsifier SGC. In spite of the neutral charge of Lipoid S75, the negative potential can be attributed to the presence of fatty acids in its composition. [33]

Table 1: PCS z-average mean particle size, polydispersity indices (PDI) and zeta potential of the donor trimyristin nanoparticles and the acceptor unilamellar vesicles.

Formulation	Z-average ± SD (nm)/PDI			Zeta potential (mV)
	Unloaded		Loaded with oxylipin at 4°C	
	Stored at 23°C	Stored at 4°C		
donor trimyristin nanoparticles	115 ± 0.6/0.14	122 ± 0.7/0.16	123 ± 0.9/0.17	-62.5 ± 1.2
Acceptor unilamellar vesicles		151 ± 1.2/ 0.07		–5.34 ± 1.1

Oxylipin transfer

The transfer of oxylipin from the donor lipid nanoparticles to the acceptor unilamellar vesicles was carried out with lipid molar ratios 1:25 and 1:100. Both molar ratios showed a very rapid initial drug transfer after 2 minutes and the steady state was reached after 33 and 35 minutes for the lipid molar ratios 1:25 and 1:100 respectively. These results show that the difference between the molar ratios was observed in the initial percent of drug transferred and the percentage of drug transferred at the steady state while the transfer rate constant was nearly the same with the different molar ratios as observed from table 2. The final amount of oxylipin transferred at both donor: acceptor molar ratios was much lower than the expected values (theoretical values) fig 2 table 2. Assuming an equal oxylipin distribution between the donor and acceptor, about 99% of the oxylipin was expected in the acceptor unilamellar vesicles at a molar ratio of 1:100 between the donor and acceptor and about 96% of the oxylipin was expected in the acceptor particles used at a molar ratio of 1:25.

Table 2: Kinetic parameters derived from fits to the transfer curves of oxylipin from the donor trimyristin solid lipid nanoparticles to the acceptor unilamellar vesicles obtained by the column method assuming transfer kinetics according to equation. [1]

Molar ratio	Transfer rate constant K (min^{-1})	Final % transferred	Equilibrium time	R^2 for fitting
1:25	0.1 ± 0.007	44 ± 2.2	33 minutes	0.998
1:100	0.12 ± 0.009	84 ± 1.3	35 minutes	0.996

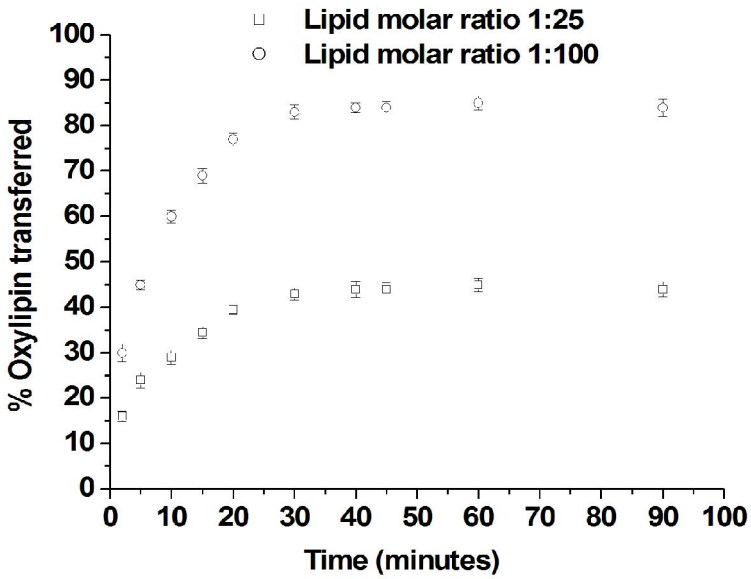

Figure 2: Percentage of oxylipin transferred from the donor solid lipid nanoparticles to the acceptor unilamellar vesicles with molar ratios of 1:25 and 1:100 obtained by the ion exchange column technique.

DISCUSSION

The oxygenated derivatives of fatty acids, known as oxylipins to which the two secondary metabolites of the bioactive fraction of *K. oryzae* belong, are important signaling molecules in animals and terrestrial plants. [34] In animal systems eicosanoids regulate cell differentiation, immune response and homeostasis. In contrast terrestrial plants use derivatives of C18 and C16 fatty acids as developmental or defense hormones. A study was conducted on the fungitoxic constituents of the basidiomycete *Gomphus floccosus* and detected 9-oxo-(10*E*, 12*E*)-octadecadienoic acids together with two other oxylipins as the active constituents with predominant antifungal activity against *Phomopsis* species. [35] The drug transfer from the colloidal lipid nanoparticles to the different acceptors, which mimics the different membranes in the body, is of great importance. Many factors affect the drug transfer from the donor particles to the different acceptors. Ion exchange column technique was used to measure the drug transfer from the lipid nanoparticles to different lipophilic acceptors that mimic the different membrane in the body. This technique was first presented by Hellings and co-workers. [36] And was after wards modified by van den Besselar and co-workers. [37] This assay employs two populations, negatively charged donor and neutral acceptor. Separation between the two populations was done on anion exchange columns, which allow the neutral acceptor to be eluted. According to this overview, this ion exchange

technique requires the use of charged particles (donor or acceptor) to separate easily the two populations. The low equilibrium values of oxylipin (steady state less than the theoretical values) might be attributed to the localization of the drug at the interface of the acceptor unilamellar vesicles (the lipophilic oxylipin did not entrapped in the vesicles bilayer). [26-28, 38] which means that after saturation of this interface the transfer stopped at low values. Additionally, the acceptor unilamellar vesicles were prepared from EPC with the addition of cholesterol, which increases the rigidity of the bilayer. [39] And occupies a part of the accessible outer surface and so decreased the amount of drug transfer to the acceptor particles.

Increasing the acceptor to donor ratio from 1:25 to 1:100 led to an increase in the final percent of drug transferred and this may be attributed to the increase in the number of the acceptor particles relative to the donor particles. This increase in the number of the acceptor particles will lead to an increase in the accessible surface available for drug transfer.

CONCLUSION

The bioactive oxylipin can be successfully incorporated into solid lipid nanoparticles. Compared to commonly applied release methods, the use of the lipophilic acceptor particles is a better approach to the conditions in blood. Ion exchange column technique can be used successfully to measure the drug transfer from the negatively charged lipid nanoparticles to the different neutral acceptors.

Declaration of interest

The authors report no declarations of interest.

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