



## FLORE

## Repository istituzionale dell'Università degli Studi di Firenze

## Improving solubility and chemical stability of natural compounds for medicinal use by incorporatng into liposomes

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Improving solubility and chemical stability of natural compounds for medicinal use by incorporating into liposomes / M.Coimbra; B.Isacchi; L.van Bloois; J.Sastre Torano; A.Ket; X.Wu; F.Broere; J.M.Metselaar; C.J:F.Rijcken; G.Storm; A.R. Bilia; R.M.Schiffelers. - In: INTERNATIONAL JOURNAL OF PHARMACEUTICS. - ISSN 0378-5173. - ELETTRONICO. - 416:(2011), pp. 433-442.

Availability:

This version is available at: 2158/594274 since: 2016-02-12T16:50:56Z

*Terms of use:* Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf)

Publisher copyright claim:

(Article begins on next page)



Contents lists available at ScienceDirect

### International Journal of Pharmaceutics



journal homepage: www.elsevier.com/locate/ijpharm

# Improving solubility and chemical stability of natural compounds for medicinal use by incorporation into liposomes

Maria Coimbra<sup>a</sup>, Benedetta Isacchi<sup>b</sup>, Louis van Bloois<sup>a</sup>, Javier Sastre Torano<sup>c</sup>, Aldo Ket<sup>a</sup>, Xiaojie Wu<sup>a</sup>, Femke Broere<sup>d</sup>, Josbert M. Metselaar<sup>a</sup>, Cristianne J.F. Rijcken<sup>a</sup>, Gert Storm<sup>a</sup>, Rita Bilia<sup>b</sup>, Raymond M. Schiffelers<sup>a,\*</sup>

<sup>a</sup> Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Dept. of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands

<sup>b</sup> Department of Pharmaceutical Sciences, University of Florence, via U. Schiff 6, 50019 Sesto Fiorentino, (FI), Italy

<sup>c</sup> Biomedical Analysis, Utrecht Institute for Pharmaceutical Sciences, Dept. of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands

<sup>d</sup> Division of Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

#### ARTICLE INFO

Article history: Received 24 November 2010 Received in revised form 24 January 2011 Accepted 25 January 2011 Available online 1 February 2011

Keywords: Caffeic acid Carvacrol Resveratrol Thymol Pterostilbene N-(3-oxo-dodecanoyl)-l-homoserine lactone Resveratrol Liposome Inflammation

### ABSTRACT

Natural bioactive compounds have been studied for a long time for their chemopreventive and therapeutic potential in several chronic inflammatory diseases, including cancer. However, their physicochemical properties generally result in poor chemical stability and lack of in vivo bioavailability. Very few human clinical trials have addressed absorption, distribution, metabolism, and excretion of these compounds in relation to efficacy. This limits the use of these valuable natural compounds in the clinic.

In this study, we examined caffeic acid (derivatives), carvacrol (derivatives), thymol, pterostilbene (derivatives), and N-(3-oxo-dodecanoyl)-l-homoserine lactone. These are natural compounds with strong anti-inflammatory properties derived from plants and bacteria. However, these compounds have poor water solubility or are chemically unstable. To overcome these limitations we have prepared liposomal formulations. Our results show that lipophilic 3-oxo-C<sub>12</sub>-homoserine lactone and stilbene derivatives can be loaded into liposomal lipid bilayer with efficiencies of 50-70%. Thereby, the liposomes solubilize these compounds, allowing intravenous administration without use of solvents. When compounds could not be loaded into the lipid bilayer (carvacrol and thymol) or are rapidly extracted from the liposomes in the presence of serum albumin  $(3- oxo-C_{12}-homoserine lactone and pterostilbene derivatives)$ , derivatization of the compound into a water-soluble prodrug was shown to improve loading efficiency and encapsulation stability. The phosphate forms of carvacrol and pterostilbene were loaded into the aqueous interior of the liposomes and encapsulation was unaffected by the presence of serum albumin. Chemical instability of resveratrol was improved by liposome-encapsulation, preventing inactivating cis-trans isomerization. For caffeic acid, liposomal encapsulation did not prevent oxidation into a variety of products. Still, by derivatization into a phenyl ester, the compound could be stably encapsulated without chemical degradation.

Despite the instability of liposome-association of 3-oxo- $C_{12}$ -homoserine lactone and resveratrol, intravenous administration of these compounds inhibited tumor growth for approximately 70% in a murine tumor model, showing that simple solubilization can have important therapeutic benefits.

© 2011 Elsevier B.V. All rights reserved.

### 1. Introduction

Nature's chemical diversity and complexity form a unique source of molecules for a wide range of therapeutic targets (Balunas and Kinghorn, 2005). Several natural compounds are long known to have chemopreventive and therapeutic properties in several chronic inflammatory diseases (Nobili et al., 2009; Pan et al., 2009). These agents generally target multiple signal transduction pathways and broadly modulate gene expression resulting in a wide-ranging spectrum of anti-inflammatory, antioxidant, immunomodulatory, pro-apoptotic, and anti-angiogenic activities (Gupta et al., 2010; Kimura, 2006; Pan et al., 2009). In the past, these agents have been chemically modified to increase the potency of the pharmacophore and increase the specificity of the molecule. At present, it seems that we are experiencing a countermovement where the natural compounds are appreciated because of their

<sup>\*</sup> Corresponding author at: Room Z735 A, Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, PO Box 80.082, 2508 TB Utrecht, The Netherlands. Tel.: +31 30 2539392; fax: +31 30 2517839.

E-mail address: R.M.Schiffelers@uu.nl (R.M. Schiffelers).

<sup>0378-5173/\$ -</sup> see front matter © 2011 Elsevier B.V. All rights reserved. doi:10.1016/j.ijpharm.2011.01.056



Fig. 1. Structural formulas of natural compounds used in this study with corresponding calculated pKa and log p values (MarvinView 5.3.2, ChemAxon Ltd.).

broad spectrum of activities making them more appropriate to interfere in multifactorial diseases, such as cancer (Balunas and Kinghorn, 2005; Harvey et al., 2010; Kimura, 2006).

In addition, compared to synthetic compounds, natural bioactive compounds generally have better safety profiles, are well accepted by the public (opinion) and are usually relatively cheap (Gupta et al., 2010). However, their physicochemical properties are generally not drug-like. In addition, the lack of human clinical trials that address their absorption, distribution, metabolism, and excretion in relation to efficacy, pose a number of challenges that need to be overcome for their establishment as effective therapeutics (Gupta et al., 2010; Nobili et al., 2009; Shoji and Nakashima, 2004).

First of all, some natural compounds tend to be biologically unstable and prone to degradation or oxidation (Shoji and Nakashima, 2004). For example, caffeic acid, an abundantly present phenolic acid in fruits, wine and coffee, is described to be rapidly oxidized into several oxidation products (Arakawa et al., 2004). Besides chemical stability, drug solubility is an issue since most natural therapeutic agents have limited water solubility (Shoji and Nakashima, 2004). Third, limited distribution to target sites and poor bioavailability are additional factors complicating the use of natural compounds in a clinical setting (Shoji and Nakashima, 2004). Finally, because of their modest potency, relatively high local drug concentrations are required for an effect. Carvacrol, thymol, resveratrol, pterostilbene, N-(3oxo-dodecanoyl)-l-homoserine lactone, caffeic acid and caffeic acid phenethyl ester are examples of natural compounds that have no or low water solubility, but have known anti-inflammatory properties (structural formulas are provided in Fig. 1).

In this study, we take advantage of liposomes to improve the solubility, stability and bioavailability of these compounds, therefore addressing the particular challenges associated with each of these natural agents.

### 2. Materials and methods

### 2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), egg-phosphatidylcholine-35 (EPC-35) and poly(ethyleneglycol)-2000distearoylphosphatidylethanolamine (PEG<sub>2000</sub>-DSPE) were a generous gift from Lipoid GmbH (Germany). Cholesterol, caffeic acid, caffeic acid phenethyl ester (CAPE), carvacrol, N-(3-oxododecanoyl)-l-homoserine lactone (3-oxo-C<sub>12</sub>-HSL), thymol, bovine albumin (BSA) in cross-linked 4% beaded agarose and trifluoroacetic acid (TFA) were provided by Sigma-Aldrich (Germany). Resveratrol was purchased from Chengdu Biopurify Phytochemicals Ltd. (China). Pterostilbene disodium phosphate and carvacrol disodium phosphate were synthesized by Syncom BV (Groningen, The Netherlands). Sodium chloride, sodium hydroxide, ammonium acetate, acetic acid and sodium dihydrogen phosphate were provided by Merck (Germany). Methanol and acetonitrile (ACN) were purchased from Biosolve (The Netherlands). Citric acid and disodium hydrogen citrate were provided by Fluka. Phosphate buffered saline (PBS) was purchased from B. Braun (Melsungen), perchloric acid (PCA) from Mallinckrodt Chemicals (Belgium), and HEPES from Acros Organics (Belgium). Methanol (MeOH; LC-MS Chromasolv), formic acid (LC–MS grade), ammonium hydroxide solution (NH<sub>4</sub>OH; 25%, LC–MS grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water was produced by a Synergy UV water delivery system from Millipore (Billerica, MA, USA).

### 2.2. Liposome preparation

### 2.2.1. Preparation of liposomes encapsulating natural compounds

Depending on the solubility of the different compounds in aqueous solutions, incorporation of the drug was either obtained in the aqueous core (caffeic acid, carvacrol phosphate, pterostilbene phosphate) or within the lipid bilayer (CAPE, carvacrol, 3-oxo- $C_{12}$ -HSL, thymol, resveratrol).

Liposomes were prepared by the lipid film hydration method. Appropriate ratios of lipids and compounds for entrapment in the bilayer were dissolved in ethanol. A lipid film was formed by rotary evaporation (Buchi, Switzerland) and flushed with a stream of nitrogen until dryness. The lipid film was hydrated at 50 °C with PBS or a solution of a natural compound in water to form liposomes. Liposome size was reduced by sequential extrusions steps (Lipex high pressure extruder, Northern Lipids) through polycarbonate membranes (Whatman, Nuclepore) of different pore sizes and with a final pore size of 100 nm or 50 nm. The mean particle size and polydispersity of the liposomal dispersions were determined by dynamic light scattering (DLS) using a Malvern ALV CGS-3 system, with a scattering angle of 90° at 25 °C. Unencapsulated drugs were removed by dialysis against PBS or 0.9% NaCl in water using a Slide-A-Lyzer cassette (Thermo Scientific, USA) with molecular weight cut-off of 10 kD at 4 °C with repeated changing of dialysis medium. Total lipid content of the liposomal dispersion was determined with a phosphate assay described by Rouser et al. (1970), performed on the organic phase after extraction of the liposomes with chloroform. Briefly, the destruction of the liposomes with PCA leads to the formation of an organic phosphate, which in turn can be quantified by a colorimetric determination. By adding a hexaammoniummolybdate solution, an organic phosphate is converted to phosphomolybdate acid which is quantitatively converted to a blue coloured compound due to reduction of ascorbic acid upon heating. The colour change can be detected using colorimetric analysis at 797 nm (Shimadzu UV mini 1240).

The amount of drug entrapped in the liposomal formulation was determined on the aqueous phase after chloroform extraction or after solubilization of the liposomes in ethanol with, either High Performance Liquid Chromatography (HPLC, Waters) or Ultra Performance Liquid Chromatography (UPLC, Waters Acquity UPLC-TUV system) using a Acquity BEH C18 1.7  $\mu$ m column (2.1 × 50 mm) (Waters).

2.2.1.1.  $3-Oxo-C_{12}$ -HSL liposomes.  $3-Oxo-C_{12}$ -HSL liposomes were prepared by entrapment in the liposomal bilayer. DPPC, PEG<sub>2000</sub>-DSPE and cholesterol were dissolved in a ratio of 1.85:0.15:1 in ethanol. Subsequently,  $3-oxo-C_{12}$ -HSL was dissolved in ethanol and added to the lipid solution to a final molar concentration of 1.7% of total lipid (TL). Liposomes were formed by film hydration with PBS and characterized as described above; dialysis was done against PBS. The amount of encapsulated  $3-oxo-C_{12}$ -HSL was quantified by UPLC using Acquity<sup>®</sup> BEH C18 1.7 µm column (2.1 × 100 mm). The mobile phase consisted of ACN, water and acetic acid (50:50:0.1 (v/v/v)).  $3-Oxo-C_{12}$ -HSL was detected using an ultraviolet detector set at a wavelength of 210 nm.

*2.2.1.2. Resveratrol liposomes.* Resveratrol liposomes were prepared by entrapment in the liposomal bilayer. DPPC, PEG<sub>2000</sub>-DSPE and cholesterol were dissolved in a ratio of 1.85:0.15:1 in ethanol. Subsequently, resveratrol was dissolved in ethanol and added to the lipid solution to prepare different liposomal dispersions with resveratrol with a final molar ratio ranging from 0.3 to 11 mol% TL. Liposomes were formed by film hydration with PBS and characterized as described above; dialysis was done against PBS. The amount of encapsulated resveratrol was quantified by HPLC using XTerra<sup>®</sup> RP18 5  $\mu$ m column (4.6 × 25 mm). The mobile phase consisted of methanol, ACN, water and acetic acid (75:22.5:2.4:0.1 (v/v/v/v)). Resveratrol was detected using an ultraviolet detector set at a wavelength of 306 nm (*trans* resveratrol) and 288 nm (*cis* resveratrol).

2.2.1.3. Carvacrol liposomes. Carvacrol liposomes were prepared by entrapment in the liposomal bilayer. DPPC,  $PEG_{2000}$ -DSPE and cholesterol were dissolved in a ratio of 1.85:0.15:1 in ethanol. Subsequently, carvacrol was dissolved in ethanol and added to the lipid solution to a final molar of 8.1%, 4% and 2% of TL. Liposomes were formed by film hydration with PBS and characterized as described above; dialysis was done against PBS. The amount of encapsulated carvacrol was quantified by UPLC using a mixture of methanol and water (25:75 (v/v)), brought to pH 2 with TFA as mobile phase. Carvacrol was detected using an ultraviolet detector set at a wavelength of 254 nm.

2.2.1.4. Thymol liposomes. Thymol liposomes were prepared by entrapment in the liposomal bilayer. EPC-35, cholesterol and PEG<sub>2000</sub>-DSPE, in a molar ratio of 1.85:1:0.15 respectively were dissolved in ethanol in a round bottom flask. Subsequently, thymol was dissolved in ethanol and added to the lipid solution to a final molar of 12.5% of TL. Liposomes were formed by film hydration with 20 mM Hepes buffer (pH 7.5), 0.8% NaCl and characterized as described above; dialysis was done against 0.9% NaCl. The liposomes were then filtrated through a 2.0 µm filter and extruded. The amount of encapsulated thymol was quantified by HPLC. The mobile phase consisted of methanol, water and PCA (75:25:0.1 (v/v/v)). Thymol was detected using an ultraviolet detector set at a wavelength of 210 nm. A sample of the liposomal dispersion was taken after preparation of multilamellar liposomes, after filtration, after extrusion and after dialysis. These samples together with a sample of the first dialysate were treated as described previously for thymol quantification by HPLC.

2.2.1.5. Carvacrol disodium phosphate liposomes. Carvacrol disodium phosphate liposomes were prepared by encapsulation in the aqueous core. DPPC, cholesterol and PEG<sub>2000</sub>-DSPE, in a molar ratio of 1.85:1.0:0.15 respectively, were dissolved in ethanol in a round-bottom flask. Liposomes were formed by film hydration with a solution of 50–100 mg/ml carvacrol disodium phosphate in water and characterized as described above; dialysis was done against 0.9% saline.

The amount of encapsulated carvacrol disodium phosphate was quantified by UPLC using a mixture of methanol and water (25:75 (v/v)), brought to pH 2 with TFA as mobile phase. Carvacrol disodium phosphate was detected using an ultraviolet detector set at a wavelength of 254 nm.

2.2.1.6. Pterostilbene disodium phosphate liposomes. Longcirculating liposomes incorporating pterostilbene phosphate disodium salt were prepared by encapsulation in the aqueous core. DPPC, cholesterol, and PEG<sub>2000</sub>-DSPE, in a molar ratio of 1.85:1.0:0.15 respectively, were dissolved in ethanol in a roundbottom flask. Liposomes were formed by film hydration of a 40 mg/ml pterostilbene phosphate in 0.9% NaCl and characterized as described above; dialysis was done against PBS. The amount of encapsulated pterostilbene phosphate was quantified by HPLC. The mobile phase consisted of ACN and water (35:65 (v/v)), brought to pH 2 with TFA. Pterostilbene phosphate was detected using an ultraviolet detector set at a wavelength of 312 nm.

2.2.1.7. Caffeic acid liposomes. Long-circulating liposomes incorporating caffeic acid were prepared by encapsulation in the aqueous core. DPPC, cholesterol, and  $PEG_{2000}$ -DSPE, in a molar ratio of 1.85:1.0:0.15 respectively, were dissolved in ethanol in a round-bottom flask. Caffeic acid was solubilized by dropwise addition of 6 M sodium hydroxide under constant stirring while controlling the pH (pH < 7.5). Liposomes were formed by film hydration of a 50 mg/ml caffeic acid solution and characterized as described above; dialysis was done against PBS.

The amount of encapsulated caffeic acid was quantified by UPLC using a mixture of methanol and water (25:75 (v/v)), brought to pH 2 with TFA as mobile phase. Caffeic acid was detected using an ultraviolet detector set at a wavelength of 324 nm.

2.2.1.8. Caffeic acid phenethyl ester (CAPE) liposomes. Two different liposome compositions containing CAPE were prepared by bilayer entrapment. EPC-35, cholesterol and PEG<sub>2000</sub>-DSPE, in a molar ratio of 1.85:1.0:0.15 respectively, were dissolved in ethanol in a round bottom flask. Subsequently, CAPE was dissolved in ethanol and added to the lipid solution to a final molar concentration of 38.6% and 20.7% of TL. Liposomes were formed by film hydration with buffered saline and characterized as described above; dialysis was done against 0.9% saline.

The amount of encapsulated CAPE was quantified by HPLC; the mobile phase consisted of ACN and water (50:50 (v/v)), with 0.1% PCA as a modifier. CAPE was detected using an ultraviolet detector set at a wavelength of 330 nm.

### 2.3. Stability studies of the liposomal formulations

To study the chemical stability of liposomal drugs at 4 °C and 37 °C, samples of liposomal 3-oxo- $C_{12}$ -HSL and liposomal resveratrol were placed in the fridge or oven and aliquots were taken at different time points. These samples were diluted 1:1 in the respective eluent for HPLC or UPLC, quantified in non-degraded drug and chromatograms were analyzed for presence of extra peaks.

The physical stability of liposomal 3-oxo- $C_{12}$ -HSL and liposomal resveratrol was studied in the presence of albumin (BSA agarose beads) for 48 h. Resveratrol and 3-oxo- $C_{12}$ -HSL liposomes were added to BSA-SepCL-4B (50%, v/v in PBS) or an equal volume of only PBS and tubes were kept in a shaking water bath at 37 °C in the dark. Aliquots were taken at multiple time points up to 48 h. Samples were centrifuged at 10,000 × g for 5 min; the supernatant was collected and stored at 2–8 °C until further analysis. The supernatant was used for the quantification of resveratrol and 3-oxo- $C_{12}$ -HSL levels by means of UPLC using the methods previously described.

The photostability of resveratrol in liposomes was studied by exposing a liposomal dispersion of resveratrol to UV light. Samples of liposomal resveratrol and resveratrol solution (in the eluent used for HPLC) were exposed to UV light in a dark cabinet (5982 mW/cm<sup>2</sup>, distance to light source 65 mm). Another sample of resveratrol solution was not exposed to UV light, but kept in the dark. Aliquots were taken after 0.1, 0.5, 1, 2, 4, 6, 8 and 16 min of UV light exposure and quantified in amount of *trans* resveratrol according to the method previously described.

The chemical and physical stability of carvacrol phosphate encapsulated liposomes was studied by incubating the liposomes at 37 °C for 96 h. At a number of time points samples of the liposomal dispersion were taken, carvacrol phosphate was quantified by means of UPLC using the method described previously and chromatograms were examined for presence of extra peaks.

### 2.4. Induction of cellular HSP70 expression by carvacrol and carvacrol disodium phosphate

To study the effect of the carvacrol derivative on mammalian APC, primary murine bone marrow derived dendritic cells (BMDC) were isolated from the bone marrow of 9-12-week-old BALB/c mice and cultured for 7 days in the presence of 10 ng ml<sup>-1</sup> GM-CSF (Cytogen) according to Lutz et al. (1999). BMDC were used on day 8 for in vitro assays. Cells were seeded in 24- or 48-well plates at 1–2 ml per well ( $2 \times 10^6$  cells ml<sup>-1</sup>). Subsequently, cells were incubated overnight with carvacrol, carvacrol-disodium phosphate (0.1 or 0.2 mM) or vehicle (ethanol at a final concentration of 0.2%) at 37 °C followed by FACS analysis of Hsp70 expression as described by Wieten et al. (2010b). To study the co-inducing capacity of carvacrol and carvacrol phosphate, the same protocol was followed, however cells were only incubated with treatments for 2 h. The plates were then sealed and placed for 1 h in a waterbath preheated at exactly 42.5 °C; cells were allowed to recover at 37 °C followed by FACS analysis.

#### 2.5. Caffeic acid stability study

Caffeic acid solutions were prepared at different pH and kept in the dark at 4 °C or 37 °C. Solutions of the maximal concentration of caffeic acid in the different buffers were used: 0.2 mg/ml in 120 mM citrate buffer pH 4, 1.6 mg/ml in 150 mM acetate buffer pH 5.5 and 150 mM phosphate buffer pH 7.2. Samples of each solution were stored for 35 days in the dark at 4 °C or 37 °C. Aliquots were taken at different time points and stored at -20 °C before quantification of caffeic acid by UPLC.

Caffeic acid degradation products were identified with HPLC coupled to mass spectrometry (MS) on an 1100 series ion trap MS equipped with an electrospray source (Agilent Technologies). Compounds were detected as negative ions and identified by their mass and fragmentation spectrum.

### 2.6. Murine tumor model

Nude Balb/c female mice (20–25 g) were obtained from Charles River and kept in standard housing with rodent chow and water available ad libitum, on a 12 h light/dark cycle. Experiments were performed in compliance with the national regulations and were approved by the local animal experiments ethical committee. The head and neck squamous-cell carcinoma was induced by subcutaneous inoculation of  $1 \times 10^6$  14C-tumor cells, a head and neck squamous-cell carcinoma line, in the flank of the mice. Tumors were measured with a digital calliper, and the tumor volume was calculated according to the formula:  $V = 1/6\pi a^2 b$ , where *a* is the smallest and b the largest superficial diameter. When tumors reached 50-100 mm<sup>3</sup>, mice were included in the study, consisting of 6 mice per treatment group. At this time, mice received 3 mg/kg liposomal 3-oxo-C<sub>12</sub>-HSL, 5 mg/kg liposomal resveratrol or equivalent dose of empty liposomes intravenously via the tail vein. Injections were repeated each 3 days. All differences between the overall effects of liposomal treatments on tumor growth were analyzed by one-way ANOVA using vehicle as a reference. A value of p < 0.05 was considered significant.

### 3. Results and discussion

In the field of drug discovery and development there has been a shift in interest from synthetic, high affinity, high potency, highly selective agents to natural (or natural-based), multiple target, low affinity and intermediate potency compounds (Harvey et al., 2010). The multi targeting properties of these natural compounds are attractive characteristics for therapies where a multitude of pathophysiological pathways are affected, like cancer (Gupta et al., 2010; Pan et al., 2009). At the same time the translation to the clinic is not straightforward as these molecules suffer from specific shortcomings. Most natural compounds have limited stability as they are prone to degradation or are highly metabolized to inactive derivates in circulation (Shoji and Nakashima, 2004). Furthermore, they generally have a poor solubility and bioavailability (Shoji and Nakashima, 2004). The use of delivery systems may help overcome these issues as reviewed recently by Li et al. for the particular case of traditional Chinese medicine or by Nair et al. for anti-inflammatory nutraceuticals for the prevention and treatment of cancer (Li et al., 2009; Nair et al., 2010). Liposomes have been extensively studied for such purposes and offer the potential to formulate both hydrophilic and hydrophobic molecules. Furthermore, liposomal encapsulation of natural compounds can increase drug solubility and stability, may allow systemic drug administration, and can enhance drug bioavailability and offer control over the absorption and distribution profiles (Shoji and Nakashima, 2004). There are several studies described in the literature, in which liposomal formulations of natural compounds were prepared to overcome solubility for in vivo application. One of the most studied natural compounds is curcumin, a principal component of turmeric, with potent biological activities but limited value as therapeutic agent due to poor water solubility and stability, which translate in low bioavailability (Teiten et al., 2010). Beyond, nanoemulsions, microspheres, solid lipid nanoparticles, polymeric nanoparticles, also liposomal formulations of curcumin were developed in order to increase its circulation time, resistance to metabolic stress and make it amenable for systemic administration (Teiten et al., 2010). Several in vitro and in vivo studies have revealed promising results with curcumin nanoparticles being more biologically active compared to free drug (Chen et al., 2009; Mach et al., 2009; Nair et al., 2010; Wang et al., 2008). In this study we have specifically addressed the issues of limited water solubility and chemical instability of a number of natural compounds by nanoformulation in liposomes.

#### 3.1. Limited solubility overcome by bilayer solubilization

3-Oxo-C<sub>12</sub>-HSL has been shown to have immune suppressive, anti-inflammatory and apoptotic effects in vitro, which can be of value for the treatment of chronic inflammatory diseases, such as cancer (Cooley et al., 2008). It is a small signalling molecule secreted by Pseudomonas aeruginosa to attenuate host immune responses and help infect pulmonary tract (Kravchenko et al., 2008). Like the majority of the natural compounds, 3-oxo-C<sub>12</sub>-HSL is not water soluble. The structural formula of this 297.4 Da molecule presents three structural segments: a 3-oxo-acyl group, a heterocyclic ring, and a side chain (Fig. 1). Despite the large apolar domain of the molecule, the resemblance of the 12 carbon acyl side chain with a (lyso)phospholipid side chain suggested that this molecule could be easily entrapped within the lipid bilayer of liposomes. Thereby solubilization for pre-clinical investigation would be enabled. A similar approach has been described for a labdane-type diterpene, which has structural similarities to cholesterol. This has led Matsingou et al. (2006) to hypothesise that this molecule would stabilize the lipid bilayer in a similar manner as the steroid. Long circulating liposomes encapsulating 3-oxo-C<sub>12</sub>-HSL were prepared with an average loading efficiency of 70%, a hydrodynamic diameter of 0.1 µm and low polydispersity (PDI < 0.1). Liposomal drug was found to be stable for up to three months at 4°C (data not shown). However, when liposomes in PBS were incubated at 37 °C, the liposome-associated drug decreased to 20% within 45 h (Fig. 2). Despite the long side chain of 3-oxo-C<sub>12</sub>-HSL which confers some resistance to ring opening, the molecule may undergo lactonolysis



**Fig. 2.** Stability of 3-oxo- $C_{12}$ -HSL and resveratrol in liposomes in the presence or absence of BSA. Liposomes containing 3-oxo- $C_{12}$ -HSL (black) and resveratrol (white) were incubated in the dark with (squares) or without (circles) BSA agarose beads in PBS for up to 48 h at 37 °C. Results are presented as average percentage of initial liposome-associated intact drug  $\pm$  SD.

by raising the temperature from 22 °C to 37 °C (Yates et al., 2002). It has been described before a decrease in quorum-sensing activity to 50% after incubation at 37 °C in cell culture medium (Ritchie et al., 2005). In the presence of bovine serum albumin (immobilized on agarose beads), the decrease in intact drug associated with liposomes was even more pronounced and may be due to presence of an acceptor for lipophilic molecules such as BSA (Fig. 2). The reversible nature of molecule-BSA binding allows transport of hydrophobic molecules in circulation and its release, making BSA a natural carrier for hydrophobic molecules (Kragh-Hansen, 1981). Within 24 h, all the 3-oxo-C<sub>12</sub>-HSL was either degraded or bound to albumin. These results imply that the liposomes can act as solubilizers for 3-oxo-C<sub>12</sub>-HSL enabling intravenous drug administration. This is important as this compound has not been pharmaceutically formulated, as yet.

In a similar set-up we studied resveratrol. Resveratrol is a natural stilbene that exhibits a strong antioxidant activity and inhibition of hydroperoxidase, protein kinase C, Bcl-2 phosphorylation, Akt, focal adhesion kinase, NF- $\kappa$ B, and matrix metalloprotease-9 (Kraft et al., 2009). A number of in vitro and in vivo studies revealed multiple intracellular targets of resveratrol which affect cell growth, inflammation, apoptosis, angiogenesis, invasion and metastasis and show that effects are dose-dependent (Fulda, 2010; Juhasz et al., 2010; Kelkel et al., 2010; Kristl et al., 2009). All these potential therapeutic applications of resveratrol are hampered by its low solubility, weak absorption after oral administration and extensive metabolism (Walle et al., 2004). Liposomes were prepared with different amounts of resveratrol in the lipid film. Maximum incorporation was  $\sim$ 5 mg/µmol TL, which was reached when 10 mg/µmol TL was added (Fig. 3). This formulation was used



Fig. 3. Liposomal association of resveratrol depending on initial drug-lipid ratio.



**Fig. 4.** Influence of carvacrol and its derivative on cellular Hsp70 expression. Primary mouse bone marrow-derived dendritic cells (BMDCs) were incubated overnight at 37 °C with carvacrol (0.1 mM or 0.2 mM), carvacrol disodium phosphate (0.1 mM or 0.2 mM) or vehicle (0.2% ethanol), followed by FACS analysis of intracellular inducible Hsp70 expression. To assess the co-inducing Hsp70 expression capacity of both treatments, cells were incubated with drugs for 2-h at 37 °C, followed by 1 h of heat shock at 42.5 °C and overnight recovery at 37 °C. Histograms are representative for at least 2 independent experiments.

for subsequent studies. Incubation in buffered saline showed that liposomal drug was stable for 28 days at 4°C (data not shown). Also at 37°C, more than 95% of intact resveratrol was retained within the liposomes (Fig. 2). Unfortunately, similar as for 3-oxo-C<sub>12</sub>-homoserine lactone, resveratrol was rapidly extracted from the liposomes upon incubation at 37°C in the presence of BSA, reaching ~10% after 24 h (Fig. 2). It has been shown by Cao et al. (2009) that the existence of strong electrostatic binding forces between resveratrol and BSA occur in a spontaneous thermodynamic process. This fact, together with the preferential localization of resveratrol at the liposomal surface, suggests that resveratrol is extracted from the lipid bilayer to form BSA-resveratrol complexes. Therefore, similar to 3-oxo-C<sub>12</sub>-HSL, liposomal formulation of resveratrol is primarily active as solubilizer.

### 3.2. Limited solubility overcome by derivatization into a water-soluble prodrug

The solubilization strategy was also tested for other natural compounds. Liposomal formulations of carvacrol and its isomer thymol could circumvent difficulties related to the lipophilicity to improve their biological activities. Carvacrol and thymol are found in many essential oils, are recognized as safe food additives and have been extensively studied, mainly for their antimicrobial and antioxidant activities (Baser, 2008). Liposomes were prepared by adding the compounds to the lipid film. Three different formulations of carvacrol were prepared with final molar ratio of 0.125, 0.063 and 0.31 drug:total lipid. Only liposomes with the lowest content of carvacrol could be extruded. At higher carvacrol contents the liposomal structure was disrupted by interaction with the lipids, similarly as the compound disrupts the microbial

cytoplasmatic membrane (Ultee et al., 2000). This restricted the maximum amount encapsulated, with a final drug/lipid ratio of 0.002 (<0.1 mg/ml). Similar results were reported by Liolios et al. (2009), which show that only 4.16% (0.045 mg/ml) of the starting amount of carvacrol could be incorporated in liposomes. In the case of thymol, the quantification of the liposomal dispersion showed that only 6% (0.25 mg/ml) of the initial thymol was present in the final liposomal dispersions (data not shown). The quantitative analyses of thymol during the different steps of liposomal preparation shows that most of the drug content (80%) is lost during the dialysis step for removal of free drug, which could be recovered from the dialysate, partially as crystals (data not shown). In contrast to 3-oxo-C<sub>12</sub>-HSL and resveratrol, the solubility of carvacrol and thymol could not be increased by liposomes.

As an alternative, a phosphate derivative of carvacrol was synthesized in an attempt to increase its solubility and thereby entrapment in the aqueous core of long-circulating liposomes. The preparation of synthetic analogues of natural compounds is known to be a solution for solubility, potency and bioavailability limitations. For example, a water-soluble prodrug of  $d-\gamma$ -tocopherol was synthesized to overcome the insolubility and oxidation of d-y-tocopherol (Takata et al., 2002). Carvacrol disodium phosphate liposomes were prepared by lipid film re-hydration followed by extrusion and were found to be  $0.12\,\mu m$  in diameter and monodisperse (PDI < 0.1). The increase in hydrophilicity resulted in successful encapsulation in the liposomal core with a final carvacrol phosphate concentration of 6 mg/ml. Furthermore, a stability study at 37 °C for 100 h indicated that the carvacrol derivative was stably entrapped with particle size remaining constant during the study, without any extra peaks appearing in the chromatograms (data not shown). A risk of modifying the structure of a pharmacophore is that it may compromise its biological activity (Mastelic et al., 2008; Torres de Pinedo et al., 2007). As shown by Ultee et al. (2002) the phenolic hydroxyl group of carvacrol is essential for its antimicrobial effect. To study whether the biological activity of carvacrol is maintained after derivatization, we assessed the capacity of both compounds to induce cellular Hsp70 expression in vitro (Fig. 4). Carvacrol is a newly identified co-inducer of Hsp70 and may, by amplification of the Hsp70-specific regulatory T cell response, protect against the development of chronic inflammatory diseases (Wieten et al.). Overnight incubation of primary BMDC at 37 °C with both carvacrol and carvacrol disodium phosphate did not influence the expression of Hsp70, as compared to vehicle incubated cells (Fig. 4). This results have already been described before for carvacrol, and this study shows that also carvacrol phosphate seems not to induce cell stress alone (Wieten et al., 2010a). Previous studies showed that carvacrol amplifies HSP70 expression induced by a bona fide stress signal, such as heat shock (Wieten et al., 2010b). When cells were incubated for 2 h with 0.1 mM of the same compounds, followed by heat shock at 42.5 °C, carvacrol phosphate co-induced the expression of Hsp70 in BMDC to the same extent as carvacrol (Fig. 4). Furthermore, our results suggest that the capability of Hsp70 co-induction by carvacrol phosphate is concentration dependent, which was not observed with the parent compound. These results show that the derivatization of carvacrol with a phosphate group does not cause loss of biological activity; in fact it may improve its biological activities. Furthermore, the high drug levels reached in the final formulation are suitable for further therapeutic in vivo evaluation and the long-circulating property of liposomes will likely increase local drug concentrations which may translate in improved therapeutic effect. This will be subject of future investigations.

One of the most studied derivatives of resveratrol is a dimethylated analog, pterostilbene, which has been shown to have stronger biological activities over the parental compound (Fulda, 2010). However, the structural differences between both compounds lead to a higher lipophilicity of pterostilbene compared to resveratrol. In order to increase the solubility of pterostilbene, a phosphate disodium salt derivative was synthesized. Pterostilbene phosphate encapsulating liposomes were prepared by hydration of the lipid film with a solution containing 100 mg/ml pterostilbene phosphate in 0.9% NaCl. Liposomes were found to be 0.12  $\mu$ m in diameter, monodisperse (PDI < 0.1) and contain 10 mg/ml pterostilbene phosphate (10% encapsulation efficiency). Indeed, these results suggest that creating a phosphate derivative of a lipophilic natural compound is a valuable strategy to overcome the low water solubility and to allow pre-clinical investigation of therapeutic applicabil-



**Fig. 5.** Chemical stability of resveratrol associated liposomes at 37 °C. Resveratrol solution and liposomal dispersion were exposed to UV light. Levels of *trans* resveratrol present were monitored up to 16 min of UV light exposure. Results are presented as percentage of initial *trans* resveratrol  $\pm$  SD.

ity. Furthermore, besides the encapsulation of the molecule in the aqueous core of the liposomes which renders more stability to the system, liposomes have long circulating property which may translate in high local dose concentrations in vivo.

### 3.3. Poor chemical stability overcome by derivatization or lipid protection

Liposomal encapsulation of natural compounds reduces drug contact and reactivity with the environment and thus may protect drugs from light and other types of degradation (Habib and Asker, 1991; Monfardini and Veronese, 1998). For example curcumin, known to degrade rapidly in buffer solutions at 37 °C (84% degraded after 180 min), is fully protected by encapsulation in liposomes at 37 °C for 180 min (Chen et al., 2009; Wang et al., 1997). Resveratrol is also relatively unstable as it converts easily to its cis form under the influence of light. In general, the stilbene backbone in *cis* configuration shows increased activity, except in the case of resveratrol which trans isomer is biologically more active (Fulda, 2010; Rius et al., 2010). The liposome association of resveratrol protects its trans-cis isomerization, since the amount of intact drug (trans isomer) remains constant when liposomes are kept at 4°C (not shown) and 37°C (Fig. 5) in the dark. Lipids also protect resveratrol under UV light exposure, with 70% trans resveratrol still present after 16 min of UV light exposure compared to 10% when resveratrol is exposed in the free form (Fig. 5).



Fig. 6. Macroscopic degradation of caffeic acid. Fresh caffeic acid solution (left); caffeic acid liposomes after extrusion (middle); caffeic acid liposomes after 48 h at 4°C (right).



**Fig. 7.** Degradation products of caffeic acid. Base peak chromatogram of a degraded caffeic acid solution (0.2 mg/ml) containing dihydroxybenzaldehyde (1), caffeic acid ortho-quinone (2), caffeic acid (3) and caffeic acid tetramers (4–7).

The required therapeutic doses of caffeic acid are high, and due to its poor solubility and, more importantly, chemical instability, its practical application in the clinic appears limited. Caffeic acid is an attractive natural compound, with biological effects ranging from antioxidative and anti-inflammatory as well as antiproliferative effects (Jiang et al., 2005). Therefore, to circumvent caffeic acid's chemical instability and to improve solubility, the formulation into liposomes seemed to be a logical approach. Liposomes were prepared by lipid film re-hydration method. By DLS the mean particle size of the liposomes prepared was found to be 0.1 µm and monodisperse (PDI < 0.1). However, liposomes prepared at physiological pH acquired a brown colour over time, which suggests that liposomes do not prevent caffeic acid oxidation (Fig. 6). Indeed, HPLC-MS/MS analysis of caffeic acid samples at pH 6.1 revealed several degradation products (Fig. 7). The main degradation product, caffeic acid ortho-quinone, is responsible for the browning of degrading caffeic acid solutions and is formed by oxidation of the phenolic groups of caffeic acid (Cilliers and Singleton, 1990; Pierpoint, 1966). Several structures of tetramers could be observed in the chromatogram. The polymeric forms of caffeic acid eluted after the caffeic acid as reported by Lutter et al. (2007). Furthermore, trace amounts of the oxidation product dihydroxybenzaldehyde were found in the samples, which can be a contributor to the polymerization of phenolic compounds. In conclusion, LC-MS data shows that the oxidation of caffeic acid leads to the formation of several oxidation products and renders such formulation unsuitable for further studies. Additional stability studies with caffeic acid show that when it is stored at low pH and temperature, caffeic acid is less prone to oxidation (Fig. 8). However, at such pH values, the solubility of caffeic is low which turns the liposome preparation of no beneficial value since these drug levels would not translate in a potent therapeutic activity. Similar as resveratrol and its naturally occurring derivative pterostilbene, there are natural derivatives of caffeic acid. Caffeic acid phenethyl ester (CAPE) is a major component of propolis extract, described in the literature as having similar or even stronger biological activities than caffeic acid (Chung et al., 2004). CAPE is more stable i.e. less prone to oxidation. One of the few studies examining the in vitro and in vivo stability of CAPE, showed that it is enzymatically hydrolyzed to caffeic acid in rat plasma, but not in human plasma (Celli et al., 2007).



**Fig. 8.** Caffeic acid stability at different pH and temperatures. Caffeic acid solutions prepared at different pH were kept at 4 °C (top) and 37 °C (bottom) for up to 34 days. Results are presented as average percentage of initial intact caffeic acid.

However CAPE is also sparingly soluble in water. Once more, in an attempt to increase CAPE solubility, two different long-circulating liposomes encapsulating CAPE were prepared. Similar to 3-oxo- $C_{12}$ -HSL, CAPE could be quantitatively entrapped within the lipid bilayer, with encapsulation efficiency of ~90%. The stable encapsulation of CAPE into liposomes allows further in vivo investigation by systemic administration, without the need of organic solvents.

### 3.4. Overcoming low bioavailability of natural compounds translates into therapeutic effects in vivo

To assess the effects of 3-oxo-C<sub>12</sub>-HSL on subcutaneous head and neck squamous-cell carcinoma growth, mice received intravenous injections of 3 mg 3-oxo-C12-HSL or 5 mg liposomal resveratrol per kg bodyweight or an equivalent dose of lipid at the moment tumors reached 50 mm<sup>3</sup>. The solubilization of the compounds in liposomes allowed intravenous drug administration and did not cause apparent toxicity. Tumor volumes were significantly smaller for 3-oxo-C<sub>12</sub>-HSL and resveratrol as compared to vehicletreated controls (p < 0.05, Fig. 9). In a study by Narayanan et al. (2009), a liposomal resveratrol formulation was used to improve bioavailability after oral gavage administration of 50 mg/kg in a genetically modified mouse model for prostate cancer. Liposomal resveratrol inhibited tumor growth by approximately 50% in this study. Others had similar results using solid lipid nanoparticles, zinc-pectinate or micelles to solubilize the compound (Das et al., 2010; Lu et al., 2009; Teskac and Kristl, 2010).

Besides the resistance to *cis-trans* isomerization by liposome encapsulation, the intravenous administration of resveratrol is likely to increase in vivo resveratrol concentrations leading to dif-



**Fig. 9.** Antitumor effect of liposomal 3-oxo- $C_{12}$ -HSL and resveratrol. Head and neck squamous-cell carcinoma-bearing mice received 3 mg/kg liposomal 3-oxo- $C_{12}$ -HSL, 5 mg/kg resveratrol (RSV) or an equivalent dose of liposomes (vehicle) when tumor size reached 50 mm<sup>3</sup> (day 0). Comparison of area-under-the-tumor-growth-curves showed that tumor proliferation was decelerated after liposomal drug treatments compared to vehicle control (\*p < 0.05, one-way ANOVA).

ferent metabolism and different pleiotropic effects that ultimately translate in an effective antitumor response (Calamini et al., 2010).

### 4. Conclusions

Taken together, these results show that many poorly soluble natural compounds can be incorporated into liposomes. Compounds that are in the bilayer tend to be extracted in the presence of albumin, limiting the function of the liposome to that of a solubilizing excipient, simply allowing drug delivery. So far, the in vivo studies show that this may still be a valuable approach to obtain therapeutic benefits. However, if drugs can be stably encapsulated into long circulating liposomes, a targeted drug delivery can take place, which may further enhance biological activities of the compounds.

#### Acknowledgments

The research of MC and RMS is supported by grant UFA 07947 of the Technology Foundation/Netherlands Organization for Scientific Research-Innovational Research Incentives Scheme VIDI.

The authors want to express their gratitude to Lipoid GmbH (Ludwigshafen, Germany) for their generous gift of DPPC and EPC-35.

#### References

- Arakawa, R., Yamaguchi, M., Hotta, H., Osakai, T., Kimoto, T., 2004. Product analysis of caffeic acid oxidation by on-line electrochemistry/electrospray ionization mass spectrometry. J. Am. Soc. Mass Spectrom. 15, 1228–1236.
- Balunas, M.J., Kinghorn, A.D., 2005. Drug discovery from medicinal plants. Life Sci. 78, 431–441.
- Baser, K.H., 2008. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. Curr. Pharm. Des. 14, 3106–3119.
- Calamini, B., Ratia, K., Malkowski, M.G., Cuendet, M., Pezzuto, J.M., Santarsiero, B.D., Mesecar, A.D., 2010. Pleiotropic mechanisms facilitated by resveratrol and its metabolites. Biochem. J. 429, 273–282.
- Cao, S., Wang, D., Tan, X., Chen, J., 2009. Interaction between *trans*-resveratrol and serum albumin in aqueous solution. J. Solution Chem. 38, 1193–1202.
- Celli, N., Dragani, L.K., Murzilli, S., Pagliani, T., Poggi, A., 2007. In vitro and in vivo stability of caffeic acid phenethyl ester, a bioactive compound of propolis. J. Agric. Food Chem. 55, 3398–3407.
- Chen, C., Johnston, T.D., Jeon, H., Gedaly, R., McHugh, P.P., Burke, T.G., Ranjan, D., 2009. An in vitro study of liposomal curcumin: stability, toxicity and biological activity in human lymphocytes and Epstein-Barr virus-transformed human B-cells. Int. J. Pharm. 366, 133–139.
- Chung, T.W., Moon, S.K., Chang, Y.C., Ko, J.H., Lee, Y.C., Cho, G., Kim, S.H., Kim, J.G., Kim, C.H., 2004. Novel and therapeutic effect of caffeic acid and caffeic acid phenyl

ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism. FASEB J. 18, 1670–1681.

- Cilliers, J.J.L., Singleton, V.L., 1990. Autoxidative phenolic ring opening under alkaline conditions as a model for natural polyphenols in food. J. Agric. Food Chem. 38, 1797–1798.
- Cooley, M., Chhabra, S.R., Williams, P., 2008. N-acylhomoserine lactone-mediated quorum sensing: a twist in the tail and a blow for host immunity. Chem. Biol. 15, 1141–1147.
- Das, S., Ng, K.-Y., Ho, P., 2010. Formulation and optimization of zinc-pectinate beads for the controlled delivery of resveratrol. AAPS PharmSciTech 11, 729–742.
- Fulda, S., 2010. Resveratrol and derivatives for the prevention and treatment of cancer. Drug Discov. Today 15, 757–765.
- Gupta, S.C., Kim, J.H., Prasad, S., Aggarwal, B.B., 2010. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastasis Rev. 29, 405–434.
- Habib, M.J., Asker, A.F., 1991. Photostabilization of riboflavin by incorporation into liposomes. J. Parenter. Sci. Technol. 45, 124–127.
- Harvey, A.L., Clark, R.L., Mackay, S.P., Johnston, B.F., 2010. Current strategies for drug discovery through natural products. Expert Opin. Drug Discov. 5, 559–568.
- Jiang, R.W., Lau, K.M., Hon, P.M., Mak, T.C., Woo, K.S., Fung, K.P., 2005. Chemistry and biological activities of caffeic acid derivatives from *Salvia miltiorrhiza*. Curr. Med. Chem. 12, 237–246.
- Juhasz, B., Varga, B., Gesztelyi, R., Kemeny-Beke, A., Zsuga, J., Tosaki, A., 2010. Resveratrol: a multifunctional cytoprotective molecule. Curr. Pharm. Biotechnol..
- Kelkel, M., Jacob, C., Dicato, M., Diederich, M., 2010. Potential of the dietary antioxidants resveratrol and curcumin in prevention and treatment of hematologic malignancies. Molecules 15, 7035–7074.
- Kimura, I., 2006. Medical benefits of using natural compounds and their derivatives having multiple pharmacological actions. Yakugaku Zasshi 126, 133–143.
- Kraft, T.E., Parisotto, D., Schempp, C., Efferth, T., 2009. Fighting cancer with red wine? Molecular mechanisms of resveratrol. Crit. Rev. Food Sci. Nutr. 49, 782–799.
- Kragh-Hansen, U., 1981. Molecular aspects of ligand binding to serum albumin. Pharmacol. Rev. 33, 17–53.
- Kravchenko, V.V., Kaufmann, G.F., Mathison, J.C., Scott, D.A., Katz, A.Z., Grauer, D.C., Lehmann, M., Meijler, M.M., Janda, K.D., Ulevitch, R.J., 2008. Modulation of gene expression via disruption of NF-kappaB signaling by a bacterial small molecule. Science 321, 259–263.
- Kristl, J., Teskac, K., Caddeo, C., Abramovic, Z., Sentjurc, M., 2009. Improvements of cellular stress response on resveratrol in liposomes. Eur. J. Pharm. Biopharm. 73, 253–259.
- Li, D.C., Zhong, X.K., Zeng, Z.P., Jiang, J.G., Li, L., Zhao, M.M., Yang, X.Q., Chen, J., Zhang, B.S., Zhao, Q.Z., Xie, M.Y., Xiong, H., Deng, Z.Y., Zhang, X.M., Xu, S.Y., Gao, Y.X., 2009. Application of targeted drug delivery system in Chinese medicine. J. Control. Release 138, 103–112.
- Liolios, C.C., Gortzi, O., Lalas, S., Tsaknis, J., Chinou, I., 2009. Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and in vitro antimicrobial activity. Food Chem. 112, 77–83.
- Lu, X., Ji, C., Xu, H., Li, X., Ding, H., Ye, M., Zhu, Z., Ding, D., Jiang, X., Ding, X., Guo, X., 2009. Resveratrol-loaded polymeric micelles protect cells from Abeta-induced oxidative stress. Int. J. Pharm. 375, 89–96.
- Lutter, M., Clark, A.C., Prenzler, P.D., Scollary, G.R., 2007. Oxidation of caffeic acid in a wine-like medium: production of dihydroxybenzaldehyde and its subsequent reactions with (+)-catechin. Food Chem. 105, 968–975.
- Lutz, M.B., Kukutsch, N., Ogilvie, A.L.J., Rößner, S., Koch, F., Romani, N., Schuler, G., 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods 223, 77–92.
- Mach, C.M., Mathew, L., Mosley, S.A., Kurzrock, R., Smith, J.A., 2009. Determination of minimum effective dose and optimal dosing schedule for liposomal curcumin in a xenograft human pancreatic cancer model. Anticancer Res. 29, 1895–1899.
- Mastelic, J., Jerkovic, I., Blazevic, I., Poljak-Blazi, M., Borovic, S., Ivancic-Bace, I., Smrecki, V., Zarkovic, N., Brcic-Kostic, K., Vikic-Topic, D., Muller, N., 2008. Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. J. Agric. Food Chem. 56, 3989–3996.
- Matsingou, C., Dimas, K., Demetzos, C., 2006. Design and development of liposomes incorporating a bioactive labdane-type diterpene. In vitro growth inhibiting and cytotoxic activity against human cancer cell lines. Biomed. Pharmacother. 60, 191–199.
- Monfardini, C., Veronese, F.M., 1998. Stabilization of substances in circulation. Bioconjug. Chem. 9, 418–450.
- Nair, H.B., Sung, B., Yadav, V.R., Kannappan, R., Chaturvedi, M.M., Aggarwal, B.B., 2010. Delivery of antiinflammatory nutraceuticals by nanoparticles for the prevention and treatment of cancer. Biochem. Pharmacol. 80, 1833–1843.
- Narayanan, N.K., Nargi, D., Randolph, C., Narayanan, B.A., 2009. Liposome encapsulation of curcumin and resveratrol in combination reduces prostate cancer incidence in PTEN knockout mice. Int. J. Cancer 125, 1–8.
- Nobili, S., Lippi, D., Witort, E., Donnini, M., Bausi, L., Mini, E., Capaccioli, S., 2009. Natural compounds for cancer treatment and prevention. Pharmacol. Res. 59, 365–378.
- Pan, M.H., Lai, C.S., Dushenkov, S., Ho, C.T., 2009. Modulation of inflammatory genes by natural dietary bioactive compounds. J. Agric. Food Chem. 57, 4467–4477.
- Pierpoint, W.S., 1966. The enzymic oxidation of chlorogenic acid and some reactions of the quinone produced. Biochem. J. 98, 567–580.
- Ritchie, A.J., Jansson, A., Stallberg, J., Nilsson, P., Lysaght, P., Cooley, M.A., 2005. The Pseudomonas aeruginosa quorum-sensing molecule N-3-(oxododecanoyl)-L-homoserine lactone inhibits T-cell differentiation and cytokine production by

a mechanism involving an early step in T-cell activation. Infect. Immun. 73, 1648–1655.

- Rius, C., Abu-Taha, M., Hermenegildo, C., Piqueras, L., Cerda-Nicolas, J.-M., Issekutz, A.C., Estañ, L., Cortijo, J., Morcillo, E.J., Orallo, F., Sanz, M.-J., 2010. Trans- but not cis-resveratrol impairs angiotensin-II-mediated vascular inflammation through inhibition of NF-κB activation and peroxisome proliferator-activated receptor-γ upregulation. J. Immunol. 185, 3718–3727.
- Rouser, G., Fkeischer, S., Yamamoto, A., 1970. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5, 494–496.
- Shoji, Y., Nakashima, H., 2004. Nutraceutics and delivery systems. J. Drug Target. 12, 385–391.
- Takata, J., Hidaka, R., Yamasaki, A., Hattori, A., Fukushima, T., Tanabe, M., Matsunaga, K., Karube, Y., Imai, K., 2002. Novel d-gamma-tocopherol derivative as a prodrug for d-gamma-tocopherol and a two-step prodrug for S-gamma-CEHC. J. Lipid Res. 43, 2196–2204.
- Teiten, M.-H., Eifes, S., Dicato, M., Diederich, M., 2010. Curcumin the paradigm of a multi-target natural compound with applications in cancer prevention and treatment. Toxins 2, 128–162.
- Teskac, K., Kristl, J., 2010. The evidence for solid lipid nanoparticles mediated cell uptake of resveratrol. Int. J. Pharm. 390, 61–69.
- Torres de Pinedo, A., Peñalver, P., Morales, J.C., 2007. Synthesis and evaluation of new phenolic-based antioxidants: structure-activity relationship. Food Chem. 103, 55–61.
- Ultee, A., Bennik, M.H., Moezelaar, R., 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Appl. Environ. Microbiol. 68, 1561–1568.

- Ultee, A., Kets, E.P., Alberda, M., Hoekstra, F.A., Smid, E.J., 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Arch. Microbiol. 174, 233–238.
- Walle, T., Hsieh, F., DeLegge, M.H., Oatis Jr., J.E., Walle, U.K., 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab. Dispos. 32, 1377–1382.
- Wang, D., Veena, M.S., Stevenson, K., Tang, C., Ho, B., Suh, J.D., Duarte, V.M., Faull, K.F., Mehta, K., Srivatsan, E.S., Wang, M.B., 2008. Liposome-encapsulated curcumin suppresses growth of head and neck squamous cell carcinoma in vitro and in xenografts through the inhibition of nuclear factor kappaB by an AKTindependent pathway. Clin. Cancer Res. 14, 6228–6236.
- Wang, Y.J., Pan, M.H., Cheng, A.L., Lin, L.I., Ho, Y.S., Hsieh, C.Y., Lin, J.K., 1997. Stability of curcumin in buffer solutions and characterization of its degradation products. J. Pharm. Biomed. Anal. 15, 1867–1876.
- Wieten, L, van der Zee, R., Goedemans, R., Sijtsma, J., Serafini, M., Lubsen, N.H., van Eden, W., Broere, F., 2010a. Hsp70 expression and induction as a readout for detection of immune modulatory components in food. Cell Stress Chaperones 15, 25–37.
- Wieten, L., van der Zee, R., Spiering, R., Wagenaar-Hilbers, J., van Kooten, P., Broere, F., van Eden, W., 2010b. A novel heat-shock protein coinducer boosts stress protein Hsp70 to activate T cell regulation of inflammation in autoimmune arthritis. Arthritis Rheum. 62, 1026–1035.
- Yates, E.A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S.R., Sockett, R.E., Goldner, M., Dessaux, Y., Camara, M., Smith, H., Williams, P., 2002. Nacylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect. Immun. 70, 5635–5646.