Original Paper

Fucoxanthin Induced Suicidal Death of Human Erythrocytes

Marilena Briglia^{a,b} Salvatrice Calabró^{a,b} Elena Signoretto^d Kousi Alzoubi^a
Stefan Laufer^c Caterina Faggio^b Florian Lang^a

^aDepartment of Physiology, University of Tübingen, Germany; ^bDepartment of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, S. Agata-Messina, Italy; ^cDepartment of Pharmacy, University of Tübingen, Germany; ^dDepartment of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milano, Italy

Key Words

Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background/Aims: Fucoxanthin, a carotenoid isolated from brown seaweeds, induces suicidal death or apoptosis of tumor cells and is thus considered for the treatment or prevention of malignancy. In analogy to apoptosis of nucleated cell, erythrocytes may enter eryptosis, the suicidal death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress and activation of p38 kinase or protein kinase C. The present study explored, whether and how fucoxanthin induces eryptosis. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, hemolysis from hemoglobin release, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, and abundance of reactive oxygen species (ROS) from DCFDA dependent fluorescence and lipid peroxidation using BODIPY fluorescence. **Results:** A 48 hours exposure of human erythrocytes to fucoxanthin significantly increased the percentage of annexin-V-binding cells ($\geq 50 \mu\text{M}$), significantly decreased average forward scatter ($\geq 25 \mu\text{M}$), significantly increased hemolysis ($\geq 25 \mu\text{M}$), significantly increased Fluo3-fluorescence ($\geq 50 \mu\text{M}$), significantly increased lipid peroxidation, but did not significantly modify DCFDA fluorescence. The effect of fucoxanthin on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+} , and was insensitive to p38 kinase inhibitor skepinone ($2 \mu\text{M}$) and to protein kinase C inhibitor calphostin (100 nM). **Conclusion:** Fucoxanthin triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to stimulation of Ca^{2+} entry.

© 2015 The Author(s)
Published by S. Karger AG, Basel

M. Briglia and S. Calabró contributed equally and thus share first authorship

Prof. Dr. Florian Lang

Physiologisches Institut der Universität Tübingen

Gmelinstr. 5, D-72076 Tübingen (Germany)

Tel. +49 7071 29 72194, Fax +49 7071 29 5618, E-Mail florian.lang@uni-tuebingen.de

Introduction

Fucoxanthin, a carotenoid from the chloroplasts of brown seaweeds [1-3] and/or its metabolite fucoxanthinol [1] have anti-mutagenic [1], anti-obesity [1-8], anti-diabetic [1, 3, 5, 6, 8], antioxidant [3, 4, 7, 8], anti-inflammatory [1, 4, 8], liver, skin, bone & eye protective [3, 8], cardio- & cerebrovascular protective [3, 4, 8], anti-angiogenic [8], and antimalarial [8] activities.

Fucoxanthinol and to a lesser extent fucoxanthin triggers apoptosis [1, 9-29] and is thus considered for the treatment or prevention of malignancy [1, 4, 7, 8, 15-17, 21, 22, 25, 28-31]. Signaling involved in the effect of fucoxanthin(ol) include NF- κ B [1, 21, 24-27, 30], p53 [10], caspases [1], Bcl-2 proteins [1, 30], MAPK [1, 30], PI3K/Akt [1, 23, 26, 27], JAK/STAT [1, 28], AP-1 [1], GADD45 [1, 30], increase of cytosolic Ca^{2+} activity [20], and caspases [10, 12, 15-19, 23, 25, 29, 30]. The substance is further partially effective by stimulating autophagy [14] and by depolarizing mitochondria [5, 6, 27]. On the other hand, partially due to its antioxidant activity, fucoxanthin may inhibit apoptosis [32, 33].

Even though lacking mitochondria and nuclei, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage [34] and by phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface [35]. Eryptosis is stimulated by Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress [35], ceramide [36], energy depletion [35], caspases [35, 37, 38], stimulation of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [35], as well as pharmacological or genetic knockout of AMP activated kinase AMPK, cGMP-dependent protein kinase, and PAK2 kinase and sorafenib/sunitinib sensitive kinases [35]. Eryptosis is stimulated by a wide variety of xenobiotics [35, 39-63] and is enhanced in diverse clinical conditions, such as dehydration [52], hyperphosphatemia [62] chronic kidney disease (CKD) [44, 64-66], hemolytic-uremic syndrome [67], diabetes [68], hepatic failure [69], malignancy [35], sepsis [70], sickle-cell disease [35], beta-thalassemia [35], Hb-C and G6PD-deficiency [35], as well as Wilsons disease [71].

The present study explored whether and how fucoxanthin triggers eryptosis. To this end, human erythrocytes from healthy volunteers were exposed for 48 hours to fucoxanthin and phosphatidylserine surface abundance, cell volume as well as $[\text{Ca}^{2+}]_i$ and ROS formation quantified by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 g for 20 min at 21 °C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl_2 , at 37°C for 24 h. Where indicated, erythrocytes were exposed to fucoxanthin (Sigma Aldrich, Hamburg, Germany), skepinone [72], or calphostin (Cayman, Ann Arbor, USA) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 100 μ l cell suspension was washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. The annexin-V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

Hemolysis

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) after incubation under the respective experimental conditions and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed once in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μ l Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 100 μ l suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnellendorf, Germany) in PBS containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed in PBS. The DCFDA-loaded erythrocytes were resuspended in 200 μ l Ringer solution, and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Lipid peroxidation

For detection of lipid peroxidation BODIPY 581/591 C11 (Thermo Fisher Scientific, MA, USA) was used. The nonoxidized dye has an emission wavelength of 590 nm (FL2) and loses fluorescence to 510 nm (FL1) upon interaction with peroxyl radicals. Following the respective treatment, cells were loaded with 5 μ M BODIPY for 30 min at 37°C in the dark. Cells were collected and analyzed by FACS Calibur (BD) at emission wavelengths of 530 nm and 585 nm.

Thiazole Orange

For studying the effect of fucoxanthin on the reticulocyte sub-population thiazole orange was used according to methods described previously [73]. Briefly, treated samples were stained with 1 μ g/ml Thiazole orange for 30 min in the dark at 37°C and measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Analysis of the plot of forward scatter vs. fluorescence of the stained samples enables the discrimination of reticulocytes from the rest of erythrocyte population, thereafter gates were set on the two population and binding to Annexin-V- Alexa Fluor™ 647 (Thermo Fisher Scientific, MA, USA) and forward scatter was determined for each.

Statistics

Data are expressed as arithmetic means $\pm$ SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. *n* denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored the putative effect of fucoxanthin on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Annexin-V-binding was determined by flow cytometry in order to identify phosphatidylserine exposing erythrocytes. Prior to measurement, erythrocytes were incubated for 48 hours in Ringer solution without or with fucoxanthin (25 - 75 μ M). As illustrated in Fig. 1, a 48 hours exposure to fucoxanthin

Fig. 1. Effect of fucoxanthin on phosphatidylserine exposure.

A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 75 μ M fucoxanthin.

B. Arithmetic means $\pm$ SEM (n = 14) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of fucoxanthin (25 - 75 μ M).

C. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 75 μ M fucoxanthin. D. Arithmetic means $\pm$ SEM (n = 4) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of fucoxanthin (25 - 75 μ M). * (p<0.05), *** (p<0.001) indicates significant difference from the absence of fucoxanthin (ANOVA).

Fig. 2. Effect of fucoxanthin on erythrocyte forward scatter.

A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 75 μ M fucoxanthin.

B. Arithmetic means $\pm$ SEM (n = 14) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) fucoxanthin (25 - 75 μ M).

C. Arithmetic means $\pm$ SEM (n = 14) of the percentage erythrocytes with forward scatter (FSC) < 200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) fucoxanthin (25- 75 μ M). D. Arithmetic means $\pm$ SEM (n = 14) of the percentage erythrocytes with forward scatter (FSC) > 800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) fucoxanthin (25- 75 μ M). ** (p<0.01), *** (p<0.001) indicate significant difference from the absence of fucoxanthin (ANOVA).

Fig. 3. Effect of fucoxanthin on hemolysis. Arithmetic means $\pm$ SEM ($n = 10$) of the percentage hemolysed erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of fucoxanthin (25 - 75 μ M). **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of fucoxanthin (ANOVA).

increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 50 μ M fucoxanthin. Similarly, fucoxanthinol treatment for 48 hours increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 25 μ M.

Cell volume was estimated by determination of forward scatter with flow cytometry. Prior to measurements the erythrocytes were again incubated for 48 hours in Ringer solution without or with fucoxanthin (25 - 75 μ M). As shown in Fig. 2, fucoxanthin decreased the average erythrocyte forward scatter, an effect reaching statistical significance at 50 μ M fucoxanthin concentration. Inspection of the histogram reveals, however, that a subpopulation of erythrocytes underwent cell shrinkage but that another subpopulation of erythrocytes underwent cell swelling. Accordingly, exposure of erythrocytes to fucoxanthin was followed by an increase of the percentage of both small (Fig. 2C) and large (Fig. 2D) erythrocytes, alterations reaching significance at fucoxanthin concentrations of 25 μ M for small erythrocytes and 50 μ M for large erythrocytes. In order to explore whether fucoxanthin exerts opposing effects on reticulocytes and on mature erythrocytes, thiazole orange was utilized in order to identify reticulocytes. As a result, fucoxanthin increased the percentage reticulocytes binding annexin-V from $15.6 \pm 1.8\%$ to $25.1 \pm 1.7\%$ ($n = 9$) and the percentage of mature erythrocytes binding annexin-V from $6.7 \pm 1.3\%$ to $16.0 \pm 0.9\%$ ($n = 9$). Fucoxanthin decreased forward scatter in reticulocytes from 810 ± 46 to 634 ± 32 ($n = 9$) and in mature erythrocytes from 716 ± 19 to 320 ± 33.6 ($n = 9$). The respective values were not significantly different between reticulocytes and mature erythrocytes.

Further experiments addressed the putative effect of fucoxanthin on hemolysis. To this end, the hemoglobin concentration in the supernatant was determined by photometry. As illustrated in Fig. 3, a 48 hours incubation of erythrocytes in the presence of fucoxanthin was followed by a significant increase of the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 25 μ M fucoxanthin concentration.

Fluo3-fluorescence was quantified in order to estimate cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). As illustrated in Fig. 4, a 48 hours exposure to fucoxanthin increased the Fluo3-fluorescence, an effect reaching statistical significance at 50 μ M fucoxanthin. In order to test whether fucoxanthin-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 75 μ M fucoxanthin in the presence or nominal absence of extracellular Ca^{2+} . As shown in Fig 5, removal of extracellular Ca^{2+} significantly blunted the effect of fucoxanthin on annexin-V-binding. However, fucoxanthin significantly increased the percentage of annexin-V-binding erythrocytes even in the absence extracellular Ca^{2+} . Thus, fucoxanthin-induced cell membrane scrambling was partially but not completely due to entry of extracellular Ca^{2+} .

In order to quantify reactive oxygen species (ROS) abundance, 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence was determined utilizing flow cytometry. As a result, the ROS abundance was similar following a 48 hours exposure in the absence of fucoxanthin (18.2 ± 0.7 a.u., $n = 10$) and in the presence of 25 μ M (16.5 ± 0.9 a.u., $n = 10$), 50 μ M (16.3 ± 1.0 a.u., $n = 10$) and 75 μ M (17.6 ± 0.9 a.u., $n = 10$) fucoxanthin. Thus, fucoxanthin did not induce appreciable oxidative stress.

Fig. 4. Effect of fucoxanthin on erythrocyte Ca^{2+} activity. A. Original histogram of Fluo3-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of fucoxanthin (75 μM). B. Arithmetic means $\pm$ SEM ($n = 14$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) fucoxanthin (25 – 75 μM). * ($p < 0.05$), ** ($p < 0.01$) indicate significant difference from the absence of fucoxanthin (ANOVA).

Fig. 5. Ca^{2+} sensitivity of fucoxanthin-induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of fucoxanthin (75 μM) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 10$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) fucoxanthin (75 μM) in the presence (left bars, + Ca^{2+}) and absence (right bars, - Ca^{2+}) of Ca^{2+} . *** ($P < 0.001$) indicates significant difference from the absence of fucoxanthin, ## ($p < 0.01$) indicate significant difference from the presence of Ca^{2+} (ANOVA).

In order to quantify lipid peroxidation, BODIPY 581/591 C11 fluorescence was determined in flow cytometry. As illustrated in Fig. 6, a 48 hours incubation of erythrocytes in the presence of fucoxanthin was followed by a significant increase of fluorescence of the oxidized form and significant decrease in the fluorescence of the nonoxidized form of BODIPY. Thus, fucoxanthin significantly increased lipid peroxidation.

To explore whether stimulation of annexin-V-binding by fucoxanthin required the activity of p38 kinase or protein kinase C, experiments were performed in the absence and presence of the p38 kinase inhibitor skepinone (2 μM) or of the protein kinase C inhibitor calphostin (100 nM). As a result, a 48 hours exposure to fucoxanthin (75 μM) increased the percentage of annexin-V-binding erythrocytes to a similar extent in the absence of the kinase inhibitors (from 2.0 ± 0.3 to 7.6 ± 0.2 %, $n = 6$), the presence of skepinone (from 2.1 ± 0.2 to 6.8 ± 0.3 %, $n = 6$), and in the presence of calphostin (from 2.1 ± 0.3 to 7.4 ± 0.5 %, $n = 6$). Thus p38 kinase or protein kinase C activities are apparently not required for the effect of fucoxanthin on erythrocyte cell membrane scrambling.

Fig. 6. Effect of fucoxanthin on lipid peroxidation. Arithmetic means $\pm$ SEM ($n = 9$) of geomean fluorescence at (A) 530 nm and (B) 585 nm emission wavelength in erythrocytes exposed for 48 hours to Ringer solution without (white bars) or with (black bars) fucoxanthin (75 μ M). ***($P < 0.001$) indicates significant difference from the absence of fucoxanthin.

Fig. 7. Dot plots of thiazole orange staining. Original dot plots of forward scatter vs thiazole orange fluorescence in erythrocytes unstained (A) and stained (B) with 1 μ g/ml thiazole orange as prescribed in materials and methods, gates R1, R2 where set to determine reticulocytes from mature erythrocytes.

Discussion

The present observations disclose a novel effect of fucoxanthin, i.e. the triggering of eryptosis, the suicidal erythrocyte death. Treatment of human erythrocytes with fucoxanthin is followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface.

The effect of fucoxanthin on cell membrane scrambling was in part due to increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), a well-known stimulator of cell membrane scrambling [35]. Accordingly, removal of extracellular Ca^{2+} significantly blunted the stimulation of cell membrane scrambling by fucoxanthin. Ca^{2+} entered presumably through Ca^{2+} permeable cation channels. Stimulators of those channels include oxidative stress [35]. However, according to DCFDA fluorescence, fucoxanthin treatment did not appreciably increase the abundance of reactive oxygen species. Moreover, stimulation of erythrocyte cell membrane scrambling by fucoxanthin did not require activation of skepinone and calphostin sensitive kinases.

Beyond its effect on cell membrane scrambling, the increase of $[\text{Ca}^{2+}]_i$ following fucoxanthin treatment is expected to activate Ca^{2+} sensitive K^+ channels with subsequent cell shrinkage due to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [34]. Accordingly, the fucoxanthin treatment was followed by a significant decrease of the average forward scatter. However, close inspection of the histogram reveals that following fucoxanthin treatment a subpopulation of erythrocytes decreases, whereas another

subpopulation of erythrocytes increases forward scatter. Thus, fucoxanthin treatment leads to swelling of a subset of erythrocytes.

The results with thiazole orange staining suggest that fucoxanthin increased the percentage of annexin-V-binding reticulocytes and mature erythrocytes. The relative effect was higher in mature erythrocytes, an observation possibly pointing to enhanced susceptibility of mature erythrocytes. Enhanced susceptibility of aged erythrocytes to oxidative stress and eryptosis has been shown previously [74].

Erythrocyte swelling may eventually result in hemolysis. Hemolysis is followed by release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules and thus occludes nephrons [75]. It is the purpose of eryptosis to clear defective erythrocytes from circulating blood prior to hemolysis [35]. Eryptosis further allows the clearance of erythrocytes infected with the malaria pathogen *Plasmodium*. In the infected host erythrocyte *Plasmodium* imposes oxidative stress, which activates the Ca^{2+} -permeable erythrocyte cation channels [35, 76]. The susceptibility of erythrocytes from carriers of sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency to eryptosis is enhanced and thus the clearance of infected erythrocytes accelerated. As a result parasitemia is decreased and the carriers of those blood disorders are partially protected against a severe course of malaria [35, 77-79]. A similar protection is provided by clinical conditions with accelerated eryptosis, such as iron deficiency [80], or by xenobiotics triggering eryptosis, such as lead [80], chlorpromazine [81] or NO synthase inhibitors [81]. If the additive effect of oxidative stress in infected erythrocytes imposed by the intracellular pathogen [35, 76] and the sensitizing effect of fucoxanthin to stimulators of eryptosis leads to the preferential eryptosis and clearance of infected erythrocytes, fucoxanthin treatment may be similarly beneficial for the clinical course of malaria.

However, excessive eryptosis may lead to anemia as soon as it is not matched by a similar increase of erythropoiesis [35]. Moreover, stimulation of eryptosis may impair microcirculation [36, 82-86] by adherence of phosphatidylserine exposing erythrocytes to the vascular wall [87], stimulation of blood clotting and triggering of thrombosis [82, 88, 89].

Conclusion

Fucoxanthin triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by and in part due to increase of cytosolic Ca^{2+} activity.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

The sponsor have had played no role in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the article for publication.

References

- 1 Martin LJ: Fucoxanthin and Its Metabolite Fucoxanthinol in Cancer Prevention and Treatment. *Mar Drugs* 2015;13:4784-4798.

- 2 Gammone MA, D'Orazio N: Anti-obesity activity of the marine carotenoid fucoxanthin. *Mar Drugs* 2015;13:2196-2214.
- 3 Muradian K, Vaiserman A, Min KJ, Fraifeld VE: Fucoxanthin and lipid metabolism: A minireview. *Nutr Metab Cardiovasc Dis* 2015;10.1016/j.numecd.2015.05.010
- 4 Kim SK, Pangestuti R: Biological activities and potential health benefits of fucoxanthin derived from marine brown algae. *Adv Food Nutr Res* 2011;64:111-128.
- 5 Maeda H: Nutraceutical effects of fucoxanthin for obesity and diabetes therapy: a review. *J Oleo Sci* 2015;64:125-132.
- 6 Miyashita K, Nishikawa S, Beppu F, Tsukui T, Abe M, Hosokawa M: The allenic carotenoid fucoxanthin, a novel marine nutraceutical from brown seaweeds. *J Sci Food Agric* 2011;91:1166-1174.
- 7 Muthuirulappan S, Francis SP: Anti-cancer mechanism and possibility of nano-suspension formulations for a marine algae product fucoxanthin. *Asian Pac J Cancer Prev* 2013;14:2213-2216.
- 8 Peng J, Yuan JP, Wu CF, Wang JH: Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: metabolism and bioactivities relevant to human health. *Mar Drugs* 2011;9:1806-1828.
- 9 Wang J, Chen S, Xu S, Yu X, Ma D, Hu X, Cao X: In vivo induction of apoptosis by fucoxanthin, a marine carotenoid, associated with down-regulating STAT3/EGFR signaling in sarcoma 180 (S180) xenografts-bearing mice. *Mar Drugs* 2012;10:2055-2068.
- 10 Wang L, Zeng Y, Liu Y, Hu X, Li S, Wang Y, Li L, Lei Z, Zhang Z: Fucoxanthin induces growth arrest and apoptosis in human bladder cancer T24 cells by up-regulation of p21 and down-regulation of mTOR. *Acta Biochim Biophys Sin (Shanghai)* 2014;46:877-884.
- 11 Das SK, Hashimoto T, Shimizu K, Yoshida T, Sakai T, Sowa Y, Komoto A, Kanazawa K: Fucoxanthin induces cell cycle arrest at G0/G1 phase in human colon carcinoma cells through up-regulation of p21WAF1/Cip1. *Biochim Biophys Acta* 2005;1726:328-335.
- 12 Das SK, Ren R, Hashimoto T, Kanazawa K: Fucoxanthin induces apoptosis in osteoclast-like cells differentiated from RAW264.7 cells. *J Agric Food Chem* 2010;58:6090-6095.
- 13 Hosokawa M, Kudo M, Maeda H, Kohno H, Tanaka T, Miyashita K: Fucoxanthin induces apoptosis and enhances the antiproliferative effect of the PPARgamma ligand, troglitazone, on colon cancer cells. *Biochim Biophys Acta* 2004;1675:113-119.
- 14 Hou LL, Gao C, Chen L, Hu GQ, Xie SQ: Essential role of autophagy in fucoxanthin-induced cytotoxicity to human epithelial cervical cancer HeLa cells. *Acta Pharmacol Sin* 2013;34:1403-1410.
- 15 Ishikawa C, Tafuku S, Kadekaru T, Sawada S, Tomita M, Okudaira T, Nakazato T, Toda T, Uchihara JN, Taira N, Ohshiro K, Yasumoto T, Ohta T, Mori N: Anti-adult T-cell leukemia effects of brown algae fucoxanthin and its deacetylated product, fucoxanthinol. *Int J Cancer* 2008;123:2702-2712.
- 16 Kim KN, Ahn G, Heo SJ, Kang SM, Kang MC, Yang HM, Kim D, Roh SW, Kim SK, Jeon BT, Park PJ, Jung WK, Jeon YJ: Inhibition of tumor growth in vitro and in vivo by fucoxanthin against melanoma B16F10 cells. *Environ Toxicol Pharmacol* 2013;35:39-46.
- 17 Kim KN, Heo SJ, Kang SM, Ahn G, Jeon YJ: Fucoxanthin induces apoptosis in human leukemia HL-60 cells through a ROS-mediated Bcl-xL pathway. *Toxicol In Vitro* 2010;24:1648-1654.
- 18 Kotake-Nara E, Asai A, Nagao A: Neoxanthin and fucoxanthin induce apoptosis in PC-3 human prostate cancer cells. *Cancer Lett* 2005;220:75-84.
- 19 Kotake-Nara E, Terasaki M, Nagao A: Characterization of apoptosis induced by fucoxanthin in human promyelocytic leukemia cells. *Biosci Biotechnol Biochem* 2005;69:224-227.
- 20 Liu CL, Huang YS, Hosokawa M, Miyashita K, Hu ML: Inhibition of proliferation of a hepatoma cell line by fucoxanthin in relation to cell cycle arrest and enhanced gap junctional intercellular communication. *Chem Biol Interact* 2009;182:165-172.
- 21 Liu CL, Lim YP, Hu ML: Fucoxanthin enhances cisplatin-induced cytotoxicity via NFkappaB-mediated pathway and downregulates DNA repair gene expression in human hepatoma HepG2 cells. *Mar Drugs* 2013;11:50-66.
- 22 Rengarajan T, Rajendran P, Nandakumar N, Balasubramanian MP, Nishigaki I: Cancer preventive efficacy of marine carotenoid fucoxanthin: cell cycle arrest and apoptosis. *Nutrients* 2013;5:4978-4989.
- 23 Rokkaku T, Kimura R, Ishikawa C, Yasumoto T, Senba M, Kanaya F, Mori N: Anticancer effects of marine carotenoids, fucoxanthin and its deacetylated product, fucoxanthinol, on osteosarcoma. *Int J Oncol* 2013;43:1176-1186.

- 24 Rwigemera A, Mamelona J, Martin LJ: Comparative effects between fucoxanthinol and its precursor fucoxanthin on viability and apoptosis of breast cancer cell lines MCF-7 and MDA-MB-231. *Anticancer Res* 2015;35:207-219.
- 25 Tafuku S, Ishikawa C, Yasumoto T, Mori N: Anti-neoplastic effects of fucoxanthin and its deacetylated product, fucoxanthinol, on Burkitt's and Hodgkin's lymphoma cells. *Oncol Rep* 2012;28:1512-1518.
- 26 Yamamoto K, Ishikawa C, Katano H, Yasumoto T, Mori N: Fucoxanthin and its deacetylated product, fucoxanthinol, induce apoptosis of primary effusion lymphomas. *Cancer Lett* 2011;300:225-234.
- 27 Ye G, Lu Q, Zhao W, Du D, Jin L, Liu Y: Fucoxanthin induces apoptosis in human cervical cancer cell line HeLa via PI3K/Akt pathway. *Tumour Biol* 2014;35:11261-11267.
- 28 Yu RX, Hu XM, Xu SQ, Jiang ZJ, Yang W: Effects of fucoxanthin on proliferation and apoptosis in human gastric adenocarcinoma MGC-803 cells via JAK/STAT signal pathway. *Eur J Pharmacol* 2011;657:10-19.
- 29 Zhang Z, Zhang P, Hamada M, Takahashi S, Xing G, Liu J, Sugiura N: Potential chemoprevention effect of dietary fucoxanthin on urinary bladder cancer EJ-1 cell line. *Oncol Rep* 2008;20:1099-1103.
- 30 Kumar SR, Hosokawa M, Miyashita K: Fucoxanthin: a marine carotenoid exerting anti-cancer effects by affecting multiple mechanisms. *Mar Drugs* 2013;11:5130-5147.
- 31 Zorofchian Moghadamtousi S, Karimian H, Khanabdali R, Razavi M, Firoozinia M, Zandi K, Abdul Kadir H: Anticancer and antitumor potential of fucoidan and fucoxanthin, two main metabolites isolated from brown algae. *ScientificWorldJournal* 2014;2014:768323.
- 32 Kaneko M, Nagamine T, Nakazato K, Mori M: The anti-apoptotic effect of fucoxanthin on carbon tetrachloride-induced hepatotoxicity. *J Toxicol Sci* 2013;38:115-126.
- 33 Zheng J, Piao MJ, Keum YS, Kim HS, Hyun JW: Fucoxanthin Protects Cultured Human Keratinocytes against Oxidative Stress by Blocking Free Radicals and Inhibiting Apoptosis. *Biomol Ther (Seoul)* 2013;21:270-276.
- 34 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca²⁺-activated K⁺ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003;285:C1553-C1560.
- 35 Lang F, Qadri SM: Mechanisms and significance of eryptosis, the suicidal death of erythrocytes. *Blood Purif* 2012;33:125-130.
- 36 Abed M, Towhid ST, Mia S, Pakladok T, Alesutan I, Borst O, Gawaz M, Gulbins E, Lang F: Sphingomyelinase-induced adhesion of eryptotic erythrocytes to endothelial cells. *Am J Physiol Cell Physiol* 2012;303:C991-999.
- 37 Lau IP, Chen H, Wang J, Ong HC, Leung KC, Ho HP, Kong SK: In vitro effect of CTAB- and PEG-coated gold nanorods on the induction of eryptosis/erythroptosis in human erythrocytes. *Nanotoxicology* 2012;6:847-856.
- 38 Maellaro E, Leoncini S, Moretti D, Del Bello B, Tanganelli I, De Felice C, Ciccoli L: Erythrocyte caspase-3 activation and oxidative imbalance in erythrocytes and in plasma of type 2 diabetic patients. *Acta Diabetol* 2013;50:489-495.
- 39 Jilani K, Lang F: Carmustine-induced phosphatidylserine translocation in the erythrocyte membrane. *Toxins (Basel)* 2013;5:703-716.
- 40 Vota DM, Maltaner RE, Wenker SD, Nesse AB, Vittori DC: Differential erythropoietin action upon cells induced to eryptosis by different agents. *Cell Biochem Biophys* 2013;65:145-157.
- 41 Zappulla D: Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO₂ increases? *J Cardiometab Syndr* 2008;3:30-34.
- 42 Lupescu A, Jilani K, Zbidah M, Lang F: Patulin-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2013;32:291-299.
- 43 Abed M, Zoubi KA, Theurer M, Lang F: Effect of dermaseptin on erythrocytes. *Basic Clin Pharmacol Toxicol* 2013;113:347-352.
- 44 Ahmed MS, Langer H, Abed M, Voelkl J, Lang F: The uremic toxin acrolein promotes suicidal erythrocyte death. *Kidney Blood Press Res* 2013;37:158-167.
- 45 Ghashghaenia M, Cluitmans JC, Toulany M, Saki M, Koberle M, Lang E, Dreischer P, Biedermann T, Duszko M, Lang F, Bosman GJ, Wieder T: Age Sensitivity of NFκB Abundance and Programmed Cell Death in Erythrocytes Induced by NFκB Inhibitors. *Cell Physiol Biochem* 2013;32:801-813.
- 46 Alzoubi K, Honisch S, Abed M, Lang F: Triggering of Suicidal Erythrocyte Death by Penta-O-galloyl-beta-D-glucose. *Toxins (Basel)* 2014;6:54-65.

- 47 Jilani K, Enkel S, Bissinger R, Almilaji A, Abed M, Lang F: Fluoxetine induced suicidal erythrocyte death. *Toxins (Basel)* 2013;5:1230-1243.
- 48 Lupescu A, Bissinger R, Jilani K, Lang F: Triggering of suicidal erythrocyte death by celecoxib. *Toxins (Basel)* 2013;5:1543-1554.
- 49 Arnold M, Lang E, Modicano P, Bissinger R, Faggio C, Abed M, Lang F: Effect of nitazoxanide on erythrocytes. *Basic Clin Pharmacol Toxicol* 2014;114:421-426.
- 50 Oswald G, Alzoubi K, Abed M, Lang F: Stimulation of suicidal erythrocyte death by ribavirin. *Basic Clin Pharmacol Toxicol* 2014;114:311-317.
- 51 Lupescu A, Bissinger R, Herrmann T, Oswald G, Jilani K, Lang F: Induction of suicidal erythrocyte death by novobiocin. *Cell Physiol Biochem* 2014;33:670-680.
- 52 Abed M, Feger M, Alzoubi K, Pakladok T, Frauenfeld L, Geiger C, Towhid ST, Lang F: Sensitization of erythrocytes to suicidal erythrocyte death following water deprivation. *Kidney Blood Press Res* 2013;37:567-578.
- 53 Alzoubi K, Calabrò S, Bissinger R, Abed M, Faggio C, Lang F: Stimulation of Suicidal Erythrocyte Death by Artesunate. *Cell Physiol Biochem* 2014;34:2232-2244.
- 54 Arnold M, Bissinger R, Lang F: Mitoxantrone-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2014;34:1756-1767.
- 55 Bissinger R, Fischer S, Jilani K, Lang F: Stimulation of Erythrocyte Death by Phloretin. *Cell Physiol Biochem* 2014;34:2256-2265.
- 56 Bissinger R, Lupescu A, Zelenak C, Jilani K, Lang F: Stimulation of eryptosis by cryptotanshinone. *Cell Physiol Biochem* 2014;34:432-442.
- 57 Bissinger R, Modicano P, Frauenfeld L, Lang E, Jacobi J, Faggio C, Lang F: Estramustine-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2013;32:1426-1436.
- 58 Jacobi J, Lang E, Bissinger R, Frauenfeld L, Modicano P, Faggio C, Abed M, Lang F: Stimulation of erythrocyte cell membrane scrambling by mitotane. *Cell Physiol Biochem* 2014;33:1516-1526.
- 59 Lupescu A, Bissinger R, Warsi J, Jilani K, Lang F: Stimulation of erythrocyte cell membrane scrambling by gedunin. *Cell Physiol Biochem* 2014;33:1838-1848.
- 60 Malik A, Bissinger R, Calabrò S, Faggio C, Jilani K, Lang F: Aristolochic Acid Induced Suicidal Erythrocyte Death. *Kidney Blood Press Res* 2014;39:408-419.
- 61 Tesoriere L, Attanzio A, Allegra M, Cilla A, Gentile C, Livrea MA: Oxysterol mixture in hypercholesterolemia-relevant proportion causes oxidative stress-dependent eryptosis. *Cell Physiol Biochem* 2014;34:1075-1089.
- 62 Voelkl J, Alzoubi K, Mamar AK, Ahmed MS, Abed M, Lang F: Stimulation of suicidal erythrocyte death by increased extracellular phosphate concentrations. *Kidney Blood Press Res* 2013;38:42-51.
- 63 Zhang R, Xiang Y, Ran Q, Deng X, Xiao Y, Xiang L, Li Z: Involvement of calcium, reactive oxygen species, and ATP in hexavalent chromium-induced damage in red blood cells. *Cell Physiol Biochem* 2014;34:1780-1791.
- 64 Abed M, Artunc F, Alzoubi K, Honisch S, Baumann D, Foller M, Lang F: Suicidal erythrocyte death in end-stage renal disease. *J Mol Med (Berl)* 2014;92:871-879.
- 65 Polak-Jonkisz D, Purzyc L: Ca(2+) influx versus efflux during eryptosis in uremic erythrocytes. *Blood Purif* 2012;34:209-210; author reply 210.
- 66 Calderon-Salinas JV, Munoz-Reyes EG, Guerrero-Romero JF, Rodriguez-Moran M, Bracho-Riquelme RL, Carrera-Gracia MA, Quintanar-Escorza MA: Eryptosis and oxidative damage in type 2 diabetic mellitus patients with chronic kidney disease. *Mol Cell Biochem* 2011;357:171-179.
- 67 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Risler T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med (Berl)* 2006;84:378-388.
- 68 Nicolay JP, Schneider J, Niemoeller OM, Artunc F, Portero-Otin M, Haik G, Jr., Thornalley PJ, Schleicher E, Wieder T, Lang F: Stimulation of suicidal erythrocyte death by methylglyoxal. *Cell Physiol Biochem* 2006;18:223-232.
- 69 Lang E, Gatidis S, Freise NF, Bock H, Kubitz R, Laueremann C, Orth HM, Klindt C, Schuier M, Keitel V, Reich M, Liu G, Schmidt S, Xu HC, Qadri SM, Herebian D, Pandya AA, Mayatepek E, Gulbins E, Lang F, Haussinger D, Lang KS, Foller M, Lang PA: Conjugated bilirubin triggers anemia by inducing erythrocyte death. *Hepatology* 2015;61:275-284.

- 70 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. *J Mol Med (Berl)* 2007;85:273-281.
- 71 Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, Koka S, Eisele K, Klarl BA, Rubben H, Schmid KW, Mann K, Hildenbrand S, Heftner H, Huber SM, Wieder T, Erhardt A, Haussinger D, Gulbins E, Lang F: Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. *Nat Med* 2007;13:164-170.
- 72 Koeberle SC, Romir J, Fischer S, Koeberle A, Schattel V, Albrecht W, Grutter C, Werz O, Rauh D, Stehle T, Laufer SA: Skepinone-L is a selective p38 mitogen-activated protein kinase inhibitor. *Nat Chem Biol* 2012;8:141-143.
- 73 Lee LG, Chen CH, Chiu LA: Thiazole orange: a new dye for reticulocyte analysis. *Cytometry* 1986;7:508-517.
- 74 Ghashghaieina M, Cluitmans JC, Akel A, Dreischer P, Toulany M, Koberle M, Skabytska Y, Saki M, Biedermann T, Duszenko M, Lang F, Wieder T, Bosman GJ: The impact of erythrocyte age on eryptosis. *Br J Haematol* 2012;157:606-614.
- 75 Harrison HE, Bunting H, Ordway NK, Albrink WS: The Pathogenesis of the Renal Injury Produced in the Dog by Hemoglobin or Methemoglobin. *J Exp Med* 1947;86:339-356.
- 76 Kirk K: Membrane transport in the malaria-infected erythrocyte. *Physiol Rev* 2001;81:495-537.
- 77 Ayi K, Giribaldi G, Skorokhod A, Schwarzer E, Prendergast PT, Arese P: 16alpha-bromoepiandrosterone, an antimalarial analogue of the hormone dehydroepiandrosterone, enhances phagocytosis of ring stage parasitized erythrocytes: a novel mechanism for antimalarial activity. *Antimicrob Agents Chemother* 2002;46:3180-3184.
- 78 Ayi K, Turrini F, Piga A, Arese P: Enhanced phagocytosis of ring-parasitized mutant erythrocytes: a common mechanism that may explain protection against falciparum malaria in sickle trait and beta-thalassemia trait. *Blood* 2004;104:3364-3371.
- 79 Cappadoro M, Giribaldi G, O'Brien E, Turrini F, Mannu F, Ulliers D, Simula G, Luzzatto L, Arese P: Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by *Plasmodium falciparum* may explain malaria protection in G6PD deficiency. *Blood* 1998;92:2527-2534.
- 80 Koka S, Huber SM, Boini KM, Lang C, Foller M, Lang F: Lead decreases parasitemia and enhances survival of *Plasmodium berghei*-infected mice. *Biochem Biophys Res Commun* 2007;363:484-489.
- 81 Koka S, Lang C, Niemoeller OM, Boini KM, Nicolay JP, Huber SM, Lang F: Influence of NO synthase inhibitor L-NAME on parasitemia and survival of *Plasmodium berghei* infected mice. *Cell Physiol Biochem* 2008;21:481-488.
- 82 Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76-82.
- 83 Closse C, Dachary-Prigent J, Boisseau MR: Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *Br J Haematol* 1999;107:300-302.
- 84 Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD, Low PS: Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. *Blood* 2003;101:4625-4627.
- 85 Pandolfi A, Di Pietro N, Siroli V, Giardinelli A, Di Silvestre S, Amoroso L, Di Tomo P, Capani F, Consoli A, Bonomini M: Mechanisms of uremic erythrocyte-induced adhesion of human monocytes to cultured endothelial cells. *J Cell Physiol* 2007;213:699-709.
- 86 Wood BL, Gibson DF, Tait JF: Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. *Blood* 1996;88:1873-1880.
- 87 Borst O, Abed M, Alesutan I, Towhid ST, Qadri SM, Foller M, Gawaz M, Lang F: Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. *Am J Physiol Cell Physiol* 2012;302:C644-C651.
- 88 Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS, Chung JH: Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. *Arterioscler Thromb Vasc Biol* 2007;27:414-421.
- 89 Zwaal RF, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005;62:971-988.