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EFFECT OF A NOVEL OMEGACOEUR®/DOLUPERINE® NUTRITIONAL COMBINATION ON HUMAN EMBRYONIC KIDNEY CELL VIABILITY

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Abstract – Holistica Laboratories (Eguilles, France) developed the nutritional supplements Omegacoeur® and Doluperine® based on two of the most ancient and unique dietary health traditions. Omegacoeur® is formulated to supply key active components of Mediterranean diet (omega 3,6,9 fatty acids, garlic, and basil) and the formulation of Doluperine® was based on the Ayurvedic tradition (curcuma, pepper, ginger extracts). Interestingly, recent studies suggest that an combination of the ingredients supplied by these two supplements could provide additional and previously unanticipated benefit through synergistic actions of some of their key components. However, the effect of such combination on human cell viability has not been investigated. In this present article, a review of the various effects of the individual compounds of the new combination and the reported active doses, and the result of a study of an combination of Omegacoeur® / Dolupérine® on Human Embryonic Kidney (HEK 293) cells. Incremental doses of 4 Omegacoeur® / Dolupérine® combinations prepared so that the molar ratio DHA (Docosahexaenoic acid) in Omegacoeur® / curcumin in Dolupérine® was kept constant, at 2.5 DHA / 1 curcumin, were added to the culture media. After 24h of incubation, cell viability was assessed by the trypan blue exclusion method. The data suggest that the combination of Omegacoeur® with Dolupérine® does not affect HEK 293 cells viability in the range of doses that have demonstrated beneficial effects in earlier studies.

Key words: Nutritional complements; Mediterranean diet; omega3- omega6- omega9-fatty-acids; garlic, piperin; ginger; curcumin; HEK 293, synergistic.

INTRODUCTION

Holistica Laboratories (Eguilles, France) develop and optimize formulas for health food supplements that are exclusively based on plants and constituents of natural origin. The fast growing body of literature available on epidemiological studies and molecular mechanism of actions of nutraceuticals support the hypothesis of a potential added benefit of a combination of two of the leading nutritional supplements developed by this company, Omegacoeur® and Doluperine®.

Abbreviations: Ωcoeur, Omegacoeur; **BSA,** Bovine serum albumin; **COX 2,** cyclooxygenase 2; **DHA,** Docosahexaenoic acid; **DMEM,** Dulbecco's Modified Eagle Medium; **Dlp,** Doluperine; **EFSA,** European Food Safety Authority; **EPA,** Eicosapentaenoic acid; **HEK,** Human Embryonic Kidney; **PUFA,** Polyunsaturated fatty acid.

Omegacoeur® and the Mediterranean diet

The Mediterranean diet has been suggested to play a beneficial role for health and longevity, and recent studies further supported previous reports that consumption of a Mediterranean diet lowers the risk of death from heart disease and cancer in both men and women (21-25,45,47). The dietary tradition of the Mediterranean region is characteristically based on a predominant consumption of fruits, vegetables, breads and cereals, nuts and seeds. Fish is also consumed on a regular basis, mostly oily fishes high in omega-3 fatty acids and widely used condiments include

garlic, onions, basil and other herbs. (6,12,14,51). Omegacoeur® is prepared with sea fish oil, Mediterranean nuxoleat (garlic/basil olive- and walnut oil maceration), and wheat germ oil. At the dosage of 4 capsules per day (for recommended nutritional or physiological support as a food supplement), Omegacoeur® supplementation provides 676 mg of omega 3 PUFA (Polyunsaturated fatty acid). This represents 572 mg of EPA (Eicosapentaenoic acid) + DHA (Docosahexaenoic acid)
which is 229 % of European Food Safety Authority (EFSA) daily value recommendations (1). Most notably, this also provides 188 % of recommended daily intake for the key component of Omégacoeur®, docosahexaenoic acid (DHA).
At an upper dosage of 6 capsules per day (recommended for health risk prevention), Omegacoeur® supplementation provides 1014 mg of omega 3 PUFA (including 858 mg of EPA + DHA : 343 % of EFSA daily value EFSA recommendations.

Doluperine® and the Ayurvedic Diet

Often referred-to as the most ancient and the foundation of major Traditional Medicines, the Indian *Ayurveda ("Science of Life")* relies heavily on the healthy effects of plants and spices and their daily used in the diet. Curcumin, one of the main pillars of Ayurveda, is thought to be responsible for most of the biological effects of turmeric, a key component of Indian curries. In support of such action, epidemiological studies have shown a lower prevalence of inflammatory diseases in the Indian population (16,43), and curcumin was shown to inhibit the key inflammatory enzymes cyclooxygenase 2 (COX 2) and lipooxygenase (8,35). It is important to note that, because of curcumin's rapid plasma clearance and conjugation, its physiological usefulness would be somewhat limited unless complexed with piperine to increase its systemic bioavailability (61) (27). Ginger is also part of the Ayurvedic health tradition. Its main uses include antimicrobial, antithrombotic, anti-inflammatory, and anticancer properties (2,70). The formula of Dolupérine® includes a turmeric extract highly concentrated in Curcumin (95%), a ginger extract titrated in gingerol (5%), and a pepper extract (95% Piperin). Two capsules of Doluperine, the recommended daily dose, provide 600 mg of curcumin in combination with 7.5 mg Piperine and 15 mg of gingerol.

The potential added benefice of an Omegacoeur® / Dolupérine® combination.

Omegacoeur® and Dolupérine® were developed based on various beneficial health effects of two clearly different sets of complementary active ingredients that have been independently incorporated into the traditional Mediterranean and Ayurvedic-based diets, respectively. With the fast-paced growth of the nutritional supplement market, a considerable effort has been put forth toward the understanding of the underlying mechanisms of action of some of the key components present in the two formulas ((26,32,42,54,59), and other refs in tables 1 and 2). Taken together, these studies revealed that distinct signalling pathways were targeted by some of the components, suggesting that key compounds of Omegacoeur® and Doluperine® could demonstrate synergistic effects. That this may indeed be the case is supported by recent *in vitro studies* and *in vivo* studies in rodents (57,69).

Clearly, the positive outcome of the above-mentioned studies warrants further investigation in humans, but the effect of the combination has not been investigated on human cell viability. Accordingly, the present study was designed to begin the assessment of the effect of the combination of Omegacoeur® and Dolupérine®, at doses chosen within the combination predicted nutritional, physiological or therapeutic range on human cell viability. The data obtained in a model of human embryonic kidney (HEK) cells suggest that the combination of Omegacoeur® with Dolupérine® did not affect HEK cell viability in a range of doses that have demonstrated beneficial effects in earlier animal studies (69).

MATERIALS AND METHODS

Doses of Omegacoeur® and Dolupérine®

DHA and curcumin are key active principles in Omegacoeur® and Dolupérine®, respectively. Accordingly, Holistica Laboratories manufacture the formulas so that the contents in DHA (in Omegacoeur®) and curcumin (in Dolupérine®) are kept constant. Hence, to determine the doses of the new Omegacoeur®/Dolupérine® combination to be tested, we choose to use the amounts of DHA present in Omegacoeur® and the amount of curcumin in Doluperine® as references. Based on dose-effects reported in the literature for the main components of both formulas (see tables 1 and 2) and previous reports of efficacy of DHA/curcumin combination (69) (57) , 4 incremental doses of the mixture were chosen for this study. They ranged from 2.5 to 2500 µM DHA in Omegacoeur® in combination with the amount of Doluperine® necessary to obtain a molar ratio of DHA / curcumin of 2.5/1.

Table 1. Selected studies of the health benefice and mechanism of action of the individual components of Omegacoeur® in major health risks.

	mg per pill of Omegacoeur ®	Cancer Risks	Neurological Risks	Cardiovascular Risks	Others
Omega 3	169 *	(17,41)	(66,68)	(29,33,40,46,58)	(11)
Omega 6	56	(10)		(34,55)	(76)
Omega 9	97	(44)		(18)	(75)
Garlic Macerate	29			(39)	(63,72)
Basil Macerate	29			(19,60)	(3)

* Includes EPA: 86 mg of eicosapentaenoic acid (EPA) and 57 mg of docosahexaenoic acid (DHA).

Table 2. Selected studies of the health benefice and mechanism of action of the individual components of Doluperine® in major health risks.

	mg per capsule of Doluperine®	Cancer Risks	Neurological Risks	Cardiovascular Risks	Others
Curcuma Extract (95% curcumin)	316 [a]	(7,53)	(4,37)	(48,49,65)	(28,30,56)
Pepper Extract (95% piperin)	4 [b]		(15)	(71)	(64,74)
Ginger Extract (5% gingerol)	150 [c]	(36)		(31,50)	(5,9,67)

[a] Includes 300 mg of curcumin, [b] includes 3.75 mg of piperine, and [c] includes 7.5 mg of gingerol.

Preparation of Omegacoeur® and Dolupérine® solutions

Fatty acids from Omegacoeur® were pre-complexed with bovine serum albumin (fatty-acid free BSA, Fisher) at the ratio of 1 mg free fatty acid / 2.6 mg Bovine serum albumin (BSA), in Dulbecco's Modified Eagle Medium (DMEM) for 18 hours at room temperature as previously described (52). Dolupérine® was prepared as a stock solution (100mM of curcumin) in 0.5M sodium hydroxide. The highest DHA/curcumin titration (2.5/1 mM) was prepared by mixing the two solutions, and contained 21 mg/ml BSA and a negligible amount of residual sodium hydroxide (1%), which did not induce any detectable change in pH of the culture media (data not shown). Three other doses were prepared by serial 1/10 dilutions from the 2.5 mM DHA/1.0 mM curcumin solution, namely 2.5/1, 25/10, and 250/100 µM DHA/curcumin ratios. To control for the potential effects of BSA, vehicle controls where Omegacoeur® and Dolupérine® were omitted were prepared in the same conditions.

Cell Culture and incubation

Human embryonic kidney 293 cells (HEK293) were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Gibco), streptomycin at 100 µg/mL, penicillin at 100 U/mL, at 37 °C in a humidified 5% CO2-containing atmosphere. HEK 293 cells were seeded (10 000-14 000 cells/cm^2) and cultured overnight in standard culture medium before addition of to the indicated Omegacoeur® and/or Dolupérine® or the corresponding vehicle control for 24h.

Morphological observation

HEK 293 cell monolayers were examined for morphological changes by inverted microscopy (Olympus IX51). Images were captured using a CCD camera connected to the microscope and computerized using the software program SPOT (Diagnostic instrument).

Cell viability

Cell viability was determined using a standard Trypan blue dye exclusion test (62). At the end of the 24hr incubation period (see above), the cells were detached by trypsin treatment and re-suspended in DMEM. Loss of cell membrane integrity was detected by classical Trypan Blue staining and viable cells (capable of excluding the dye) were counted using a haemocytometer. Data were expressed in percentage of viable cells counted after 24h of incubation in media alone.

Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM) of 4-6 independent experiments per experimental group. Treated groups were compared to the control group (media only) using paired student's t-test. *P* values < 0.05 were considered significant.

RESULTS

The Aim of the present study was to determine whether a combination of Omegacoeur® and Dolupérine® could result in a somewhat unexpected but nonetheless possible toxicity in human cells in the therapeutic range. To this end, HEK cell monolayers were exposed for 24h to a mixture of Omegacoeur® and Dolupérine®, and cell viability was assessed by the trypan blue exclusion method. To determine the doses, the amounts of DHA present in Omegacoeur® and the amount of curcumin in Doluperine® were used as references. Incremental doses for the mixture were then prepared so that the molar ratio of DHA in Omegacoeur® / curcumin in Dolupérine® was kept constant, at 2.5 DHA / 1 curcumin.

Dose effect of Omegacoeur® and Dolupérine® combination on HEK 293 viability

The effect of 24h of incubation in the presence of 4 increasing doses of the mixture and their respective vehicle controls (corresponding amount of BSA alone, as detailed in Methods) were tested. As shown in Figure 1A, the microscopic observation did not reveal any effect of the vehicle alone at any of the concentrations used. Using the trypan blue exclusion method, we confirmed quantitatively that the highest dose of BSA alone (21 mg/ml) did not have any effect on HEK cell morphology or viability (not shown). Hence, the vehicle was omitted in the subsequent studies and a control with standard culture media only was used for the quantitative analyses presented in Figures 1B and 2. At concentrations ranging from 2.5 to 250 µM DHA in Omegacoeur®, none of the Omegacoeur® / Dolupérine® combinations had a significant effect on HEK cell morphology (Fig 1A). Quantitative analysis using the trypan blue exclusion test revealed that cell viability was 118%±31, 146%±30 and 69%±20 after incubation with the 2.5/1 µM, 25/10 µM, and 250/100 µM DHA in Omegacoeur® / curcumin in Dolupérine® mixtures, respectively. None of these conditions we found significantly different from the control (100%, Fig 1B). In contrast, the higher concentration tested (2.5/1 mM DHA in Omegacoeur® / curcumin in Dolupérine®) induced a clear change in cell morphology after 24h (Fig 1A), followed by detachment and death, as revealed by the quantitative analysis by trypan blue exclusion (Fig 1B, $p<0.05$).

Effect of high concentrations of Omegacoeur® and/or Dolupérine® on HEK 293 viability

To clarify the origin of the negative effect of 24h of incubation with the Omegacoeur® / Dolupérine® mixture at the highest concentration on HEK cell viability, we next examined the individual effects of Omegacoeur® and

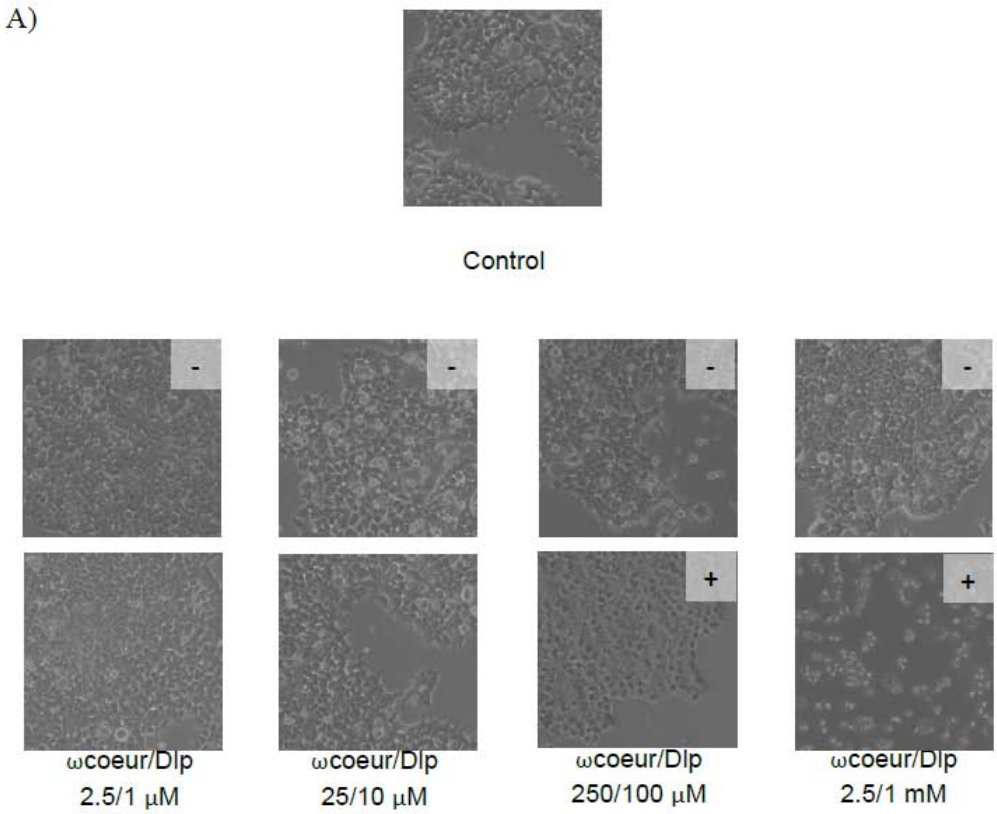

Figure 1. Dose effect of Omegacoeur®/Dolupérine® combination on HEK 293 viability. A) Microscopic observations (X200) of HEK 293 cells following 24 hours of incubation in the presence of the indicated concentrations of DHA (in Omegacoeur®)/curcumin (in Dolupérine®) combination, or in the presence of the corresponding vehicle control prepared as detailed in the "Material and Methods" section. A representative picture is shown for each dose of the combination (+) underneath a representative picture of the corresponding vehicle control (-). ***Control***: standard culture medium. ***ωcoeur/Dlp 2.5/1 µM***: 2.5 µM DHA in omegacoeur /1 µM curcumin in Doluperine. ***ωcoeur/Dlp 25/10 µM:*** 25 µM DHA in omegacoeur /10 µM curcumin in Doluperine. ***ωcoeur/Dlp 250/100 µM.*** 250 µM DHA in omegacoeur /100 µM curcumin in Doluperine. ***ωcoeur/Dlp 2.5/1 mM.*** 2.5 mM DHA in omegacoeur /1 mM curcumin in Doluperine. **B)** Quantitative analysis of HEK 293 cell viability following 24 hours of incubation in the presence of incremental concentrations of DHA (in Omegacoeur®)/curcumin (in Dolupérine®) combination prepared as indicated in the "Material and Methods" section. Data are means ± SEM of 6 independent experiments. *p<0.05 *vs.* Control (standard culture media).

Dolupérine® when given separately at those concentrations. HEK cell monolayers were exposed to 2.5mM of DHA in Omegacoeur®, 1 mM of curcumin in Dolupérine®, or a mixture of the two, and cell viability was determined using the trypan blue exclusion test after 24h. Microscopic observation revealed that HEK cell morphology was unchanged after 24h of incubation with 2.5 mM of DHA in Omegacoeur®, undistinguishable from control (Fig 2A). Consistently, cell viability was not significantly different from that of the control (81% ± 32, *NS*). On the other hand, 1 mM curcumin in Doluperine alone or in combination with 2.5 mM of DHA in Omegacoeur® induced a significant toxicity on HEK cells. Taken together, these results suggested that 1 mM curcumin in Doluperine at very high concentration accounted for the cytotoxicity observed in the mixture at high concentration (100-1000 times the health risk prevention dosages).

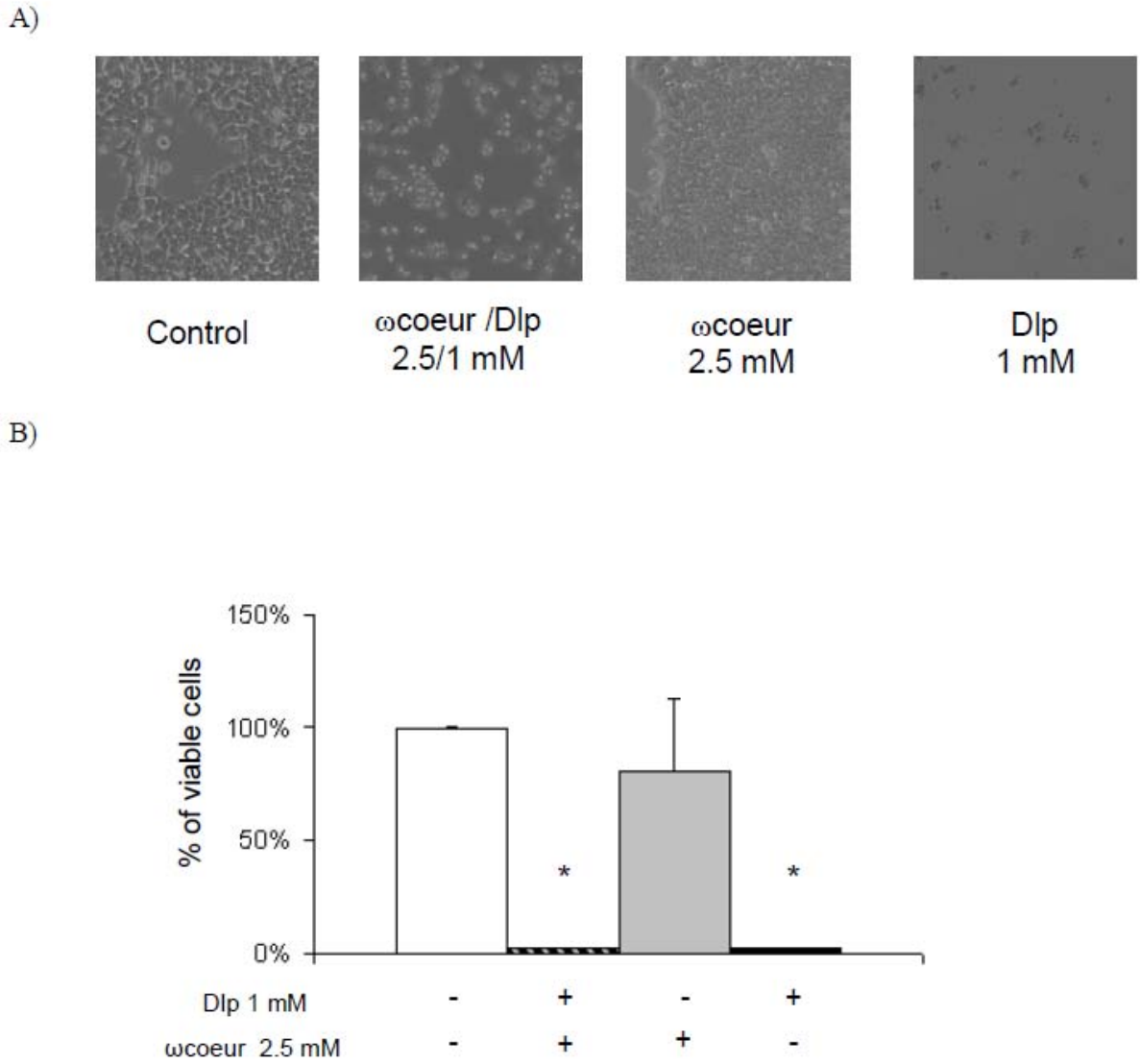

Figure 2. Effects of high concentrations of Omegacoeur®, Dolupérine®, and a 2.5/1 ratio combination on HEK 293 viability. **A)** Microscopic observations (X200) of HEK 293 cells following 24 hours of incubation with or without 2.5 mM of DHA in Omegacoeur® and/or 1 mM of curcumin in Dolupérine®. **B)** Quantitative analysis of HEK 293 viability following 24 hours of incubation with the concentration of 2.5 mM of DHA in Omegacoeur® and the concentration 1 mM of curcumin in Dolupérine® individually or in combination. Shown are means ± SEM of 6 independent experiments *p<0.05 *vs.* Control (standard culture media).

DISCUSSION

A mixture of the two nutritional supplements Omegacoeur® and Dolupérine® could provide additional benefits through synergistic actions of some of their key components, but the safety of such combination have not been examined in human cells. This study provides the first set of evidences in support of the innocuousness of the combination of Omegacoeur® and Dolupérine® in human embryonic kidney cells at active relevant doses.

The 25/10 Omegacoeur® /Dolupérine® combination is safe and well within the expected active range

The data presented in Figure 1 indicated that the 2.5/1, 25/10, and 250/100 ratios of µM DHA in Omegacoeur® / µM curcumin in Dolupérine® did not induce significant cell death in human embryonic kidney cells. Based on current literature, the 25/10 Omegacoeur® /Dolupérine® combination seems the most promising of all doses studied. It is important to keep in mind that, in addition to 25 µM DHA and 10 µM curcumin, the 25/10 mixture also contains 6.7 µM gingerol, 0.18 µM piperine, as well as Omega-6 and Omega 9 PUFA at about 8.4 µg/ml and 1.4 µg/ml, respectively. These concentrations are within the range of EC50s of well-documented beneficial effects of these components. Interistingly, DHA has been shown to reduce endothelial expression of vascular cell adhesion molecule 1 (VCAM-1), E-selectin, intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6), and IL-8 in response to IL-1, IL-4, tumor necrosis factor, or bacterial endotoxin at concentrations ranging 1-25 µM (20). Similarly, the amount of curcumin in the mixture appears suitable for anti-inflammatory action since the reported EC50 of curcumin for inhibition of epidermal lipoxygenase and cyclooxygenase activities is 5-10 µM (35). In addition to these anti-inflammatory effects, the combination of 25µM DHA and 10 µM of curcumin has been shown to suppress cell growth and stimulate apoptosis in human pancreatic cancer cell lines (69). Finally, gingerol has shown inhibitory effect on COX 2 and NF-kappaB at 5–30 µM (38). Taken together, these suggest that the 25/10 µM combination contains suitable amounts of the various compounds. Highly relevant to potential therapeutic use, those concentrations are also within achievable plasma concentrations (61). Finally, the DHA/curcumin ratio itself has been recently shown to promote synergism between DHA and curcumin in animal cells, raising the interesting possibility of achieving biological activity without toxicity at an even lower range. Indeed, Saw et al. have recently shown that an combination 0.78 µM DHA or EPA / 2.5µM curcumin has synergistic anti-inflammatory effects on murine leukemic monocytic macrophage cells (57). Clearly, further studies are warrant to further optimize the dosage of the Omegacoeur®/Dolupérine® mixture in human, but based on this brief review of the literature and the results of the present study, biological effects have been reported for doses that are at least 100 times lower than the toxic dose of the combination of Omegacoeur® and Dolupérine® (2.5/1 mM) that we observed in HEK cells.

Effect at high concentration

Because 1 mM Dolupérine® alone produced a toxicity undistinguishable from the toxicity of the combination of 2.5 mM Omegacoeur®/ 1 mM Dolupérine® (Figure 2), it is likely that one of the main components of Dolupérine® was responsible for the effect. Even though this dose of Dolupérine® includes 0.19 mg/ml ginger (0.67mM gingerol) and 0.005 mg/ml pepper (0.018 mM piperine) in addition to the 1mM curcumin and other natural curcuminoids, the toxic effect is consistent with previous reports of the effect of high doses of curcuma. Indeed, EC50 for toxicity of natural curcuminoids on HEK 293 cells was shown to be 37-200 µM after 16h (13). Since the high dose of Dolupérine® contained approximately 5 times this amount of natural curcuminoids, it is reasonable to conclude that the 1 mM curcumin along with the other natural curcuminoids in Dolupérine® were responsible for the toxic effect we observed. Finally, in favour of an absence of further toxic effect of ginger and pepper, Unnikrishnan et al. have shown an absence of toxic effect on human lymphocytes at higher doses than the ones used in the present study (ginger 0.25 mg/ml and pepper 0.1 mg/ml) (73).

In summary, this study shows, for the first time, that the combination of two nutritional supplements based on the Mediteranean diet (Omegacoeur® naturally rich in omega3,6,9 PUFA) and the Ayurvedic diet (Doluperine® enriched in curcuma, pepper, and ginger extracts) did not induce cell death in for human embryonic

kidney cells at active-relevant doses. Future preclinical studies and clinical trials shall determine whether the potential beneficial effects of this combination indeed translates into nutritional, physiological or therapeutic effects at different dosages in the prevention of health risks or as a complementary physiological support in combination with the treatments of a variety of disease processes including cancer, neurological and cardiovascular disorders.

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