Zinc ionophore activity of quercetin and epigallocatechin-gallate: From Hepa1-6 cells to a liposome model

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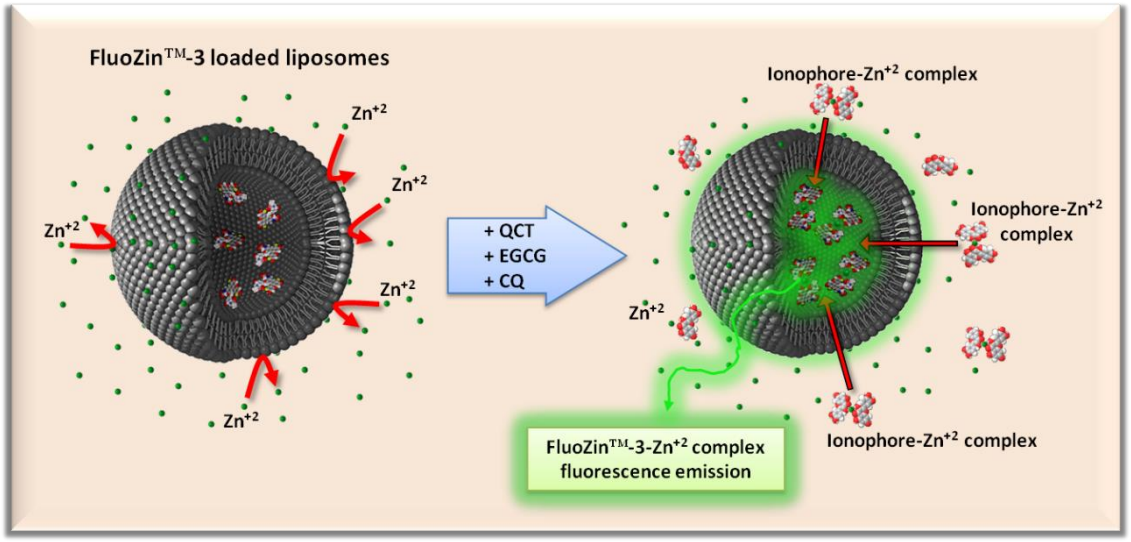
5.1. Abstract

Labile zinc, a tiny fraction of total intracellular zinc that is loosely bound to proteins and easily interchangeable, modulates the activity of numerous signaling and metabolic pathways. Dietary plant polyphenols such as the flavonoids quercetin and epigallocatechin-gallate act as antioxidants and as signaling molecules. Remarkably, the activities of numerous enzymes that are targeted by polyphenols are dependent on zinc. We have previously shown that these polyphenols chelate zinc cations and hypothesized that these flavonoids might be also acting as zinc ionophores, transporting zinc cations through the plasma membrane. To prove this hypothesis, herein, we have demonstrated the capacity of quercetin and epigallocatechin-gallate to rapidly increase labile zinc in mouse hepatocarcinoma Hepa 1-6 cells as well as, for the first time, in liposomes. In order to confirm that the polyphenols transport zinc cations across the plasma membrane independently of plasma membrane zinc transporters, quercetin, epigallocatechin-gallate, or clioquinol, alone and combined with zinc, were added to unilamellar dipalmitoylphosphocholine:cholesterol liposomes loaded with membrane-impermeant FluoZin-3. Only the combinations of the chelators with zinc triggered a rapid increase of FluoZin-3 fluorescence within the liposomes, thus demonstrating the ionophore action of quercetin, epigallocatechin-gallate, and clioquinol on lipid membrane systems. The ionophore activity of dietary polyphenols may underlay the raising of labile zinc levels triggered in cells by polyphenols and thus many of their biological actions.



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5.2. Introduction

Quercetin (QCT), a water-insoluble flavonoid present in onions, nuts, and many other vegetables, and epigallocatechin-3-gallate (EGCG), a water-soluble flavonoid present in green tea, are among the most consumed and most studied polyphenols present in the human diet.[1] Flavonoids are considered bioactive micronutrients whose regular consumption, either as food components, or as dietary supplements and nutraceuticals,[2] entails benefits for human health, including prevention and amelioration of cancers,[3] diabetes, and cardiovascular[4] and neurodegenerative[5] diseases. Many of the health benefits of flavonoids have historically been ascribed to their antioxidant activity, which they exert directly by scavenging reactive oxygen

species (ROS) and by chelating the redox-active transition metals iron and copper, which may act as ROS generators in biological systems.[6] Flavonoids also act as antioxidants indirectly by inhibiting redox-sensitive transcription factors and pro-oxidant enzymes as well as through induction of phase II and antioxidant enzymes.[7] However, it is currently believed that the levels of polyphenols achieved through ingestion are not enough to justify their wide array of biological actions. Beyond their antioxidant actions, flavonoids are also known to act as signaling molecules that, either directly or indirectly, interact with proteins and nucleic acids, thus modulating multiple cell signaling pathways, gene transcription, metabolic fluxes, and cell fate including apoptosis.[8,9]

Diverse polyphenols have been shown able to form complexes with the redox-inactive transition metal zinc.[10] Zinc is an essential micronutrient for humans, the deficiency of which causes multiple dysfunctions, including alterations of glucidic and lipidic metabolisms.[11] Within cells, the vast majority of zinc cations (in concentrations usually ranging from 100 to 300 µM for most cells) are tightly bound to proteins, functioning as a catalytic or structural component of an estimated 3000 mammalian proteins involved in virtually all cellular processes.[12] A minor fraction of intracellular zinc, termed labile zinc, exists in its free ionic form (picomolar concentrations) or loosely bound to proteins (in nanomolar concentrations). This pool of zinc is detectable by specific fluorophores with very high affinities for zinc cations at neutral pH such as Zinquin and FluoZin-3. Zinc ionophores such as pyrithione and clioquinol (CQ) have been used to increment labile zinc within cells and determine the fundamental roles that this zinc pool plays in cellular biology. Thus, free and labile zinc acts as second messenger molecule, which modulates the activity of many enzymes and thus signaling and metabolic pathways and cellular processes, including cell fate and apoptosis.[13,14] While many enzymes are inhibited by small elevations of zinc concentrations, others are activated.[15,16] Mammalian cells tightly control the subcellular distribution of zinc cations and the levels of labile zinc through the coordinated action of dedicated transmembrane zinc transporters and zinc-chelating metallothioneins.

Zinc from the extracellular milieu and from intracellular compartments enters the cytoplasm through 14 specialized transmembrane proteins of the ZIP/SLC39 family, whereas cytoplasmic extrusion of zinc toward organelles or the extracellular environment is performed by 10 transporters of the ZnT/SLC30 family, being ZnT1, located at the plasma membrane, the main regulator of cellular zinc efflux and export of excess zinc in most cells.[17] Within the cytoplasm, zinc may bind to metal free apo-metallothionein (apo-MT) to generate Zn-MT complexes. The apo-MT/Zn-MT ratio controls free and labile zinc concentrations. MT also serves as a ROS scavenger and heavy metal chelator, and the transcription of MT responds, in addition to zinc, to stress stimuli such as ROS, heavy metals, and pro-inflammatory cytokines.[18] In response to elevations of intracellular zinc, the zinc-sensor transcription factor MTF1 coordinately up-regulates the expression of MT and ZnT1,[19] thus keeping zinc levels within a functional range. Excessively high levels of labile zinc are associated with cellular death through apoptosis.[20] Dysfunctions of MT and zinc transporters are promoting factors in cardiovascular diseases,[21] diabetes,[22] Alzheimer's disease,[23] and cancer.[24]

Several studies have shown that flavonoids affect zinc metabolism. For instance, rats fed during long periods with bicalain and rutin showed reduced hepatic levels of total zinc, as well as iron and copper, implying that flavonoids may sequester these metals and render them unavailable for absorption in a similar way as phytate.[25] Consistent with this, feeding obese rats with proanthocyanidins reverse dyslipidemia and lower protein levels of ZnT1 in the liver, reflecting lower levels of hepatic zinc.[26] Early in vitro studies showed that, in human intestinal Caco-2 cells,

genistein enhanced the expression of MT, here regarded as an antioxidant enzyme,[27] while QCT increased the copper-induced expression of MT.[28] More recently, QCT was shown to enhance zinc uptake by Caco-2 cells, increasing total zinc accumulation and MT expression.[29] In contrast, grape seed flavonoids, produced a reduction in apical zinc uptake in Caco-2 cells, similar to that produced by phytate, whereas EGCG did not alter zinc absorption.[30] In prostate cancer cells, EGCG accelerated the accumulation of total zinc in the cytosol and mitochondria.[31] Two reports have shown that polyphenols may produce an increase of intracellular labile zinc. A water-soluble glycoside of the isoflavone genistin enhanced MT expression in human hepatocarcinoma HepG2 cells concomitantly increasing labile zinc and cellular death.[32] Furthermore, the stilbene resveratrol was shown to enhance total and labile zinc in normal human prostate epithelial cells, while not significantly affecting MT expression, and this was accompanied by increased cellular death. These authors suggested that the increment of labile zinc elicited by resveratrol might be due to the uptake of resveratrol-zinc complexes, followed by the dissociation of the complexes in the cytoplasm.[33]

Conversely, zinc may affect the bioactivity of flavonoids, as detailed in a few reports, including one that outlines the stimulating effect of zinc on the apoptotic effect of genistein in osteoclastic cells.[34] Zinc also yields EGCG effective in protecting cultured rat hepatocytes against hepatotoxin-induced cell injury[35] and enhances the antiproliferative, proapoptotic effects of EGCG on various lines of prostate cancer cells.[36] Zinc was also shown to affect the uptake of EGCG by prostate carcinoma cells, where Zn-EGCG chelates were less internalized by cells than EGCG alone, while mixtures of EGCG with zinc enhanced the transport of EGCG into the cells. These authors also showed that zinc enhances the incorporation of EGCG into liposomes.[37]

We have previously reported that the water-soluble flavonoid EGCG and the water-insoluble flavonoid QCT profoundly alter zinc homeostasis in cultured human and mouse hepatocarcinoma cells. Whereas EGCG reduced the levels of total intracellular zinc and the expression of MT and ZnT1,[38] QCT enhanced total zinc accumulation as well as MT and ZnT1 expression (M. Bustos, personal communication, 2011, Universitat Rovira i Virgili). However, both QCT and EGCG dose-dependently prevented zinc-induced toxicity, suggesting that most zinc cations in the culture medium are rendered unavailable to cells due to their chelation by flavonoids and the formation of flavonoid-zinc concatemers, as shown for iron and copper complexed with diverse polyphenols.[39] In addition, both polyphenols enhanced cytoplasmic levels of Zinquin-detectable labile zinc, suggesting that a fraction of the flavonoid molecules in the culture medium formed complexes with zinc that cross the plasma membrane; that is, the flavonoids may also act as zinc ionophores, transporting zinc cations across the plasma membrane independently from zinc transporters.

The aim of this work was to evaluate the capacity of QCT and EGCG to act as zinc ionophores. CQ was also tested in this study as it is a synthetic antitumor drug recently reported to induce apoptosis in diverse cells lines by enhancing intracellular labile zinc and therefore inferred to act as a water-soluble zinc ionophore.[40] We evaluated the ability of QCT, EGCG, and CQ to chelate zinc cations and the subsequent formation of a complex with FluoZin-3, a fluorophore that displays a very high affinity for zinc cations (Kd = 15 nM).[41] The uptake of zinc by mouse hepatocarcinoma cells was measured fluorescently using FluoZin-3 in the presence and absence of QCT, EGCG, and CQ. This study was repeated using unilamellar liposomes with encapsulated FluoZin-3 to investigate whether the transport of the zinc cations across the cytoplasmic membrane to form a complex with FluoZin-3 was indeed enhanced by the presence of QCT, EGCG, or CQ or was simply due to the activity of cellular zinc transporters.

5.3. Materials and methods

5.3.1. Chemicals

The lipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, zinc chloride ($ZnCl_2$), QCT, EGCG, CQ, dimethyl sulfoxide (DMSO), ethanol, and phosphate-buffered saline (0.01 M PBS, pH 7.4) were bought from Sigma-Aldrich. Cell-impermeant FluoZin-3 tetrapotassium salt and cell permeant FluoZin-3 AM were bought from Molecular Probes. A Simplicity 185 Millipore water system was used to obtain Milli-Q water (18.2 $m\Omega \cdot cm^{-1}$) for the preparation of buffers and liposomes. The compounds QCT, EGCG, and CQ were dissolved as 100 µM solutions in 100% DMSO, aliquoted, and stored at -20 °C. ZnCl2 was stored as 1 M solution in ethanol/PBS (50%/50% v/v). FluoZin-3 indicators were used at 10 µM in 100% DMSO.

5.3.2. Cell culture and treatments

The mouse hepatoma cell line Hepa 1-6 was obtained from the European Collection of Cell Cultures (BW7756 ECACC) and propagated in Dulbecco's Modified Eagle medium (DMEM; BioWittaker) supplemented with 10% fetal bovine serum (BioWittaker), 2 mM glutamine in 0.85% NaCl, 1000 U/mL penicillin/streptomycin, and 1.25 M HEPES. This medium contains 4.9 ± 0.2 µM zinc, as determined by flame atomic absorption spectroscopy (FAAS). Cells were cultured at 37 °C in a humidified, 5% CO_2-enriched atmosphere and routinely split every 3-4 days at a 1:5 ratio upon reaching approximately 80% confluence. For treatments, cells at 80% confluence were detached with Accutase (Sigma−Aldrich) and resuspended at a density of 5×10^5 cells/mL; 500 µL of this cell suspension (25×10^4 cells) was then seeded per well in 24-well plates (Orange Scientific). Twenty four hours after plating, medium was removed, and the cells were treated by adding 100 µL of fresh medium containing either 50 µM $ZnCl_2$, 100 µM QCT, 100 µM EGCG, 100 µM CQ, or the combination of 50 µM $ZnCl_2$ with each chelator for 1 and 4 h, respectively. As a control experiment, untreated cells were incubated just with medium and vehicle (final 0.1% DMSO and 0.05% ethanol).

5.3.3. Measurements of cytoplasmic labile zinc in Hepa 1-6 cells

The intracellular levels of free and labile zinc cations were measured as the fluorescence emission of cells upon loading them with the membrane-permeant zinc specific detector FluoZin-3, using fluorescence microscopy as described.[42] Briefly, following cell treatment (Section 2.2), culture media were replaced with a fresh one containing 1.5 µM FluoZin-3 (AM, cell permeant) and incubated for 30 min at 37 °C. This medium was then removed, and the cells were washed three times with PBS, and the zinc-dependent FluoZin-3 fluorescence within cells was measured using a Nikon Eclipse TE2000-S microscope, with excitation set at 494 nm and emission at 516 nm. Fluorescent intensities were quantified using the NIS-Elements AR software (Nikon Instruments) and the software ImageJ, a Java-based image processing program developed at the NIH (National Institutes of Health).[43]

5.3.4. Liposomes as cell membrane models

Homogeneous populations of liposomes were prepared using a previously reported method.[44] FluoZin-3 in a final concentration of 3 µM was mixed with 5 mL of PBS (0.01 M, pH 7.4) in a glass reactor protected from light induced degradation, under stirring conditions and a blanket of argon gas. After 15 min, a mixture of DPPC and cholesterol (9:1 molar ratio) was added and maintained under stirring conditions and argon at 25 °C for another 15 min. The homogeneous

mixture was then treated with a rapid pH jump from pH 7.4 to pH 11 and then back to pH 7.4 within a 3 s frame, followed by an equilibration step of 25 min where lipid clusters curl into FluoZin-3 encapsulating liposomes. The resulting FluoZin-3 loaded liposomes were purified using a Sephadex G-100 size-separation column and used immediately. Liposomes with encapsulated FluoZin-3 were separately incubated with 10 µM QCT, EGCG or CQ, in the presence and absence of 10 µM $ZnCl_2$. All the solutions were allowed to incubate at 25 °C for 30 min before measuring their fluorescence. For the kinetic experiment, liposomes loaded with FluoZin-3 were added to three different cuvettes, the fluorescence was measured for 15 min, followed by addition of $ZnCl_2$ (final 10 µM) to each cuvette, and the fluorescence emission was measured for another 15 min. Finally, QCT, EGCG, or CQ was added (final 10 µM) to each sample, and fluorescence emission was monitored over the duration of 1 h until the fluorescent intensity reached a plateau.

5.4. Results

5.4.1. QCT, EGCG, and CQ increase the cytoplasmic labile zinc in Hepa 1-6 cells

The increase of cytoplasmic labile zinc is modulated by the cellular zinc transporters, where the zinc ions are transported to the cytoplasm through specific channels of the ZIP family, bound to ionophore molecules that independently cross the lipid bilayer, or liberated from zinc binding proteins such as metallothioneins (Figure 5.2).

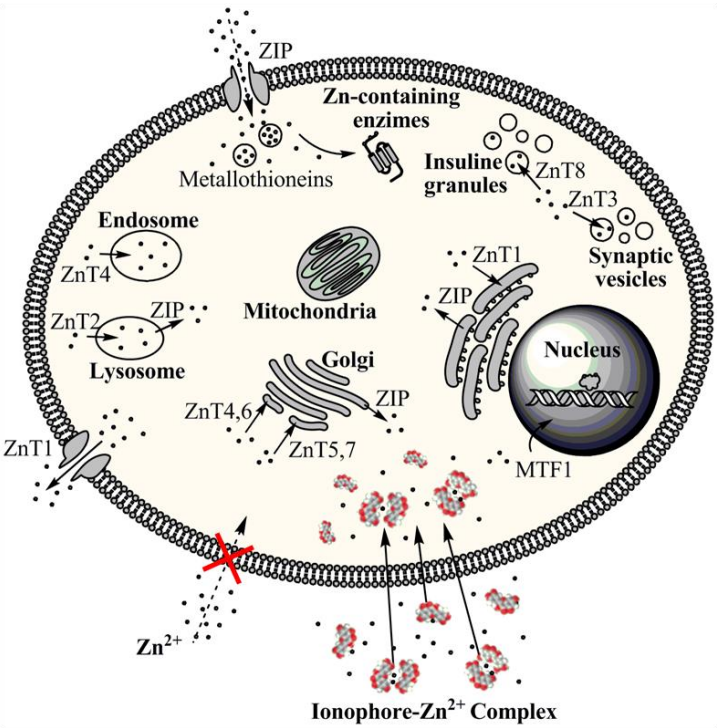

Figure 5.2. Schematic representation of zinc homeostasis. Intracellular labile zinc is modulated by the coordinated activity of a large family of zinc transporters (ZnT and ZIP) and zinc-binding proteins, such as metallothionein or ionophore molecules.

In order to assess the effect of QCT, EGCG, CQ and zinc on cytoplasmic labile zinc, Hepa 1-6 cells were treated for 1 and 4 h with the chelators and supplemental zinc, and variations in the intracellular levels of labile zinc were measured as changes in the FluoZin-3 fluorescence intensity as described in Section 2.3. Figure 5.3 shows the fluorescent images of the Hepa 1-6

cells after 1 and 4 h of treatment. No significant increase in fluorescence was obtained when QCT or EGCG were added to the culture medium without additional zinc. Only CQ insignificantly enhanced the fluorescence in these conditions, that is, with basal zinc concentration in the culture medium, which is roughly 5 µM.

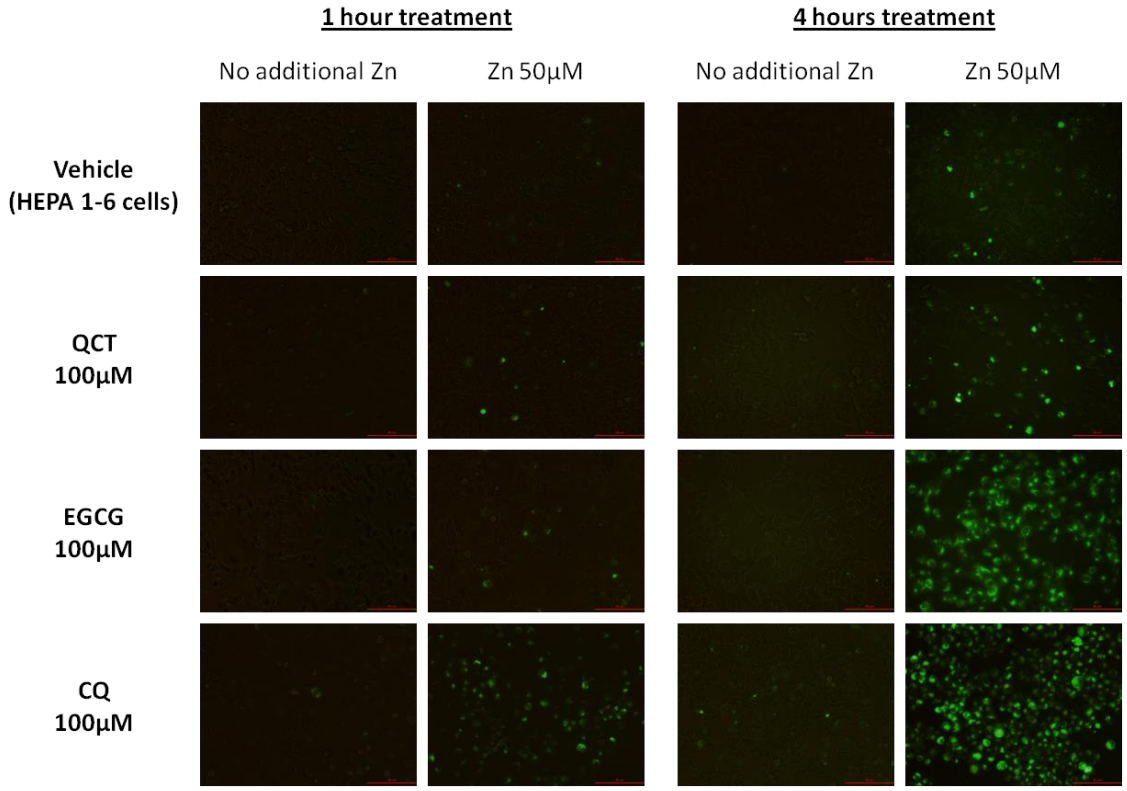

Figure 5.3. Effect of QCT, EGCG, and CQ on the cytoplasmic pool of labile zinc in Hepa 1-6 cells. Hepa 1-6 cells were first treated with 100 µM QCT, EGCG, or CQ, in the presence or absence of 50 µM $ZnCl_2$ for 1 and 4 h. The medium was then removed, and 3 µM FluoZin-3 (AM, cell permeant) was added. After 30 min incubation, cells were washed and examined using a confocal fluorescence microscope. Control cells were treated with vehicle (final 0.05% ethanol, 0.1% DMSO). Scale bars are 50 µm.

However, when 50 µM $ZnCl_2$ was added, both QCT and EGCG doubled the amount of FluoZin-3-detectable zinc after 1 h, and CQ increased this pool of zinc 10-fold with respect to the control (50 µM $ZnCl_2$ in the absence of any of QCT, EGCG or CQ) (Figure 5.4), suggesting a slower ionophore action of the flavonoids as compared to CQ. After 4 h treatment with additional 50 µM $ZnCl_2$, all treatments triggered a significant increase in fluorescence intensity. In the case of the control, the increase of the cytoplasmic labile zinc is associated with the plasma membrane ZIP transporters, whereby zinc ions are transported into the cell. QCT doubled the amount of labile zinc attained with only 50 µM zinc, EGCG quadrupled this value, and CQ increased it 7-fold.

A closer view of intracellular distribution of FluoZin-3 fluorescence (Figure 5.5) after 4 h of treatment shows a similar punctuated pattern of labile zinc for CQ, EGCG and QCT, suggesting similar ways of action for the three compounds.

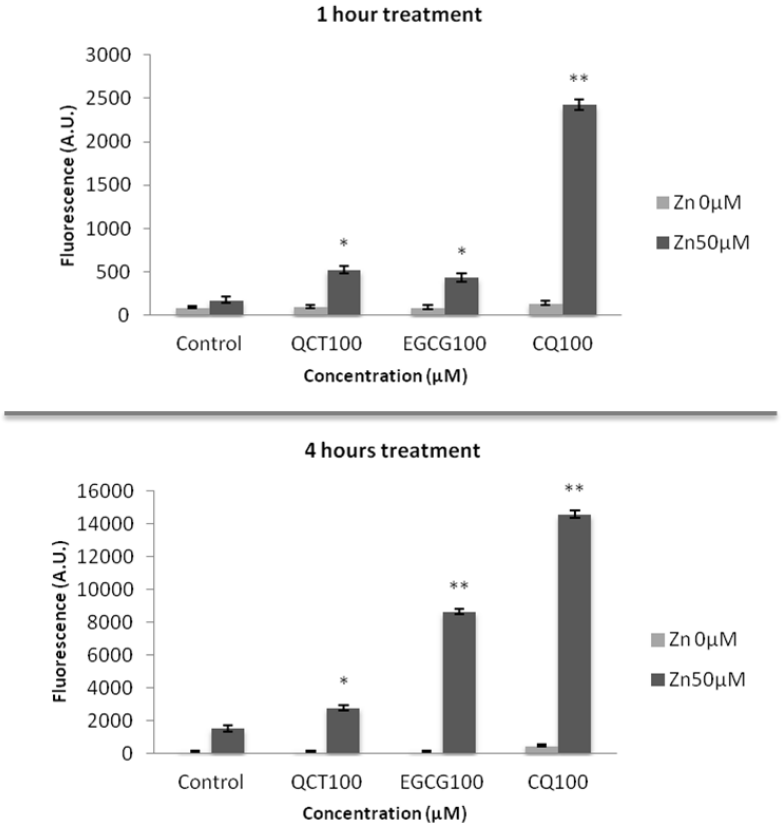

*Figure 5.4. Intensity of FluoZin-3 fluorescence signal from images in Figure 5.3 was quantified using quanta program and considering an equal number of cells in each field. All values are mean ± SEM of three independent experiments. Significant differences between treatments were determined using one-way ANOVA (Tukey test). *P ≤ 0.05; **P ≤ 0.01.*

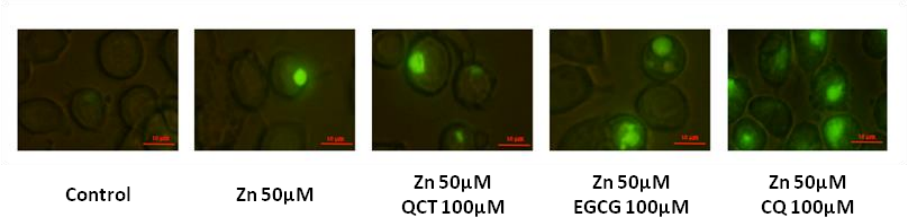

Figure 5.5. Subcellular localization of FluoZin-3 detectable zinc in Hepa 1-6 cells after 4 h treatment in the same samples as in Figure 5.3 showed at a greater magnification. Scale bars are 10 µm.

5.4.2. Zinc ionophore activity of QCT, EGCG, and CQ using liposomes as membrane models

Increases of cytoplasmic labile zinc levels triggered by CQ and pyrithione in a variety of cell lines have been attributed to their ionophore activity, that is, to the capacity of CQ-zinc and pyrithione-zinc complexes to cross the plasma membrane. To our knowledge, however, the classification of CQ and pyrithione as zinc ionophores is based on their functional effect in cells, that is, the rapid increase in Zinquin-detectable or Fluozin-3-detectable intracellular zinc, but no direct biochemical assay has been performed to discard the involvement of plasma membrane zinc importers or the origin of labile zinc from intracellular components on this effect. Furthermore, there is no report confirming that polyphenols are able to transport zinc across

the plasma membrane independently of cell transport mechanisms, such as zinc transporters or endocytosis. To directly prove the ionophore action of the flavonoids and CQ, we tested their capacity to transport zinc cations across the lipid bilayer of protein-free liposomes as a model system that mimics a cell membrane devoid of protein and polysaccharide fractions. Taking advantage of our previously reported method for the rapid preparation of liposomes,[44] 3 µM concentration of cell impermeant FluoZin-3 was encapsulated within unilamellar liposomes composed of DPPC:cholesterol in a 9:1 molar ratio. The resulting FluoZin-3-loaded liposomes were purified by passing the sample through a Sephadex G-100 size-separation column to remove the unencapsulated FluoZin-3 molecules. Dynamic light scattering (DLS) and ζ potential analysis were performed to clearly confirm the presence of stable liposomes within a size range 1−2 µm and surface charge around zero (Table 5.1).

Table 5.1. Dynamic light scattering and ζ Potential Measurements of liposomes loaded with FluoZin-3 before and after treatments with 10 µM quercetin (QCT10), 10 µM epigallocatechin-3-gallate (EGCG10) or 10 µM clioquinol (CQ10) in the presence and absence of 10 µM quercetin (QCT10), 10 µM epigallocatechin-3-gallate (EGCG10) or 10 µM clioquinol (CQ10) in the presence and absence of 10 µM zinc chloride (Zn10)[a]

Sample	Size average (µM)	ζ potential (mV)
FluoZin-3 loaded liposomes	1.4 ± 0.3	-4.7 ± 2.5
FluoZin-3 loaded liposomes + Zn10	1.1 ± 0.7	-5.0 ± 5.9
FluoZin-3 loaded liposomes + QCT10	1.2 ± 0.5	-1.9 ± 4.1
FluoZin-3 loaded liposomes + EGCG10	1.5 ± 0.4	-6.9 ± 7.6
FluoZin-3 loaded liposomes + CQ10	1.4 ± 0.2	-3.0 ± 3.7
FluoZin-3 loaded liposomes + Zn10 + QCT10	1.8 ± 0.2	-1.2 ± 2.5
FluoZin-3 loaded liposomes + Zn10 + EGCG10	1.6 ± 0.3	-3.5 ± 3.2
FluoZin-3 loaded liposomes + Zn10 + CQ10	1.4 ± 0.2	-9.0 ± 2.7

[a] Final concentrations of solvents in the samples were 0.05 % ethanol and 0.1 % DMSO. Standard deviations were calculated from the mean data of a series of experiments (n ≥ 3).

The zinc ionophore activity of polyphenols was then tested as their capacity to transport zinc cations into the liposome cavity, interacting with the encapsulated zinc-dependent FluoZin-3 and consequently increasing the fluorescence signal within the liposomes (Figure 5.6). $ZnCl_2$ (10 µM) was added to the liposomal suspension in the absence and presence of 10 µM QCT, EGCG and CQ, respectively, and zinc-dependent fluorescence intensity was measured over time.

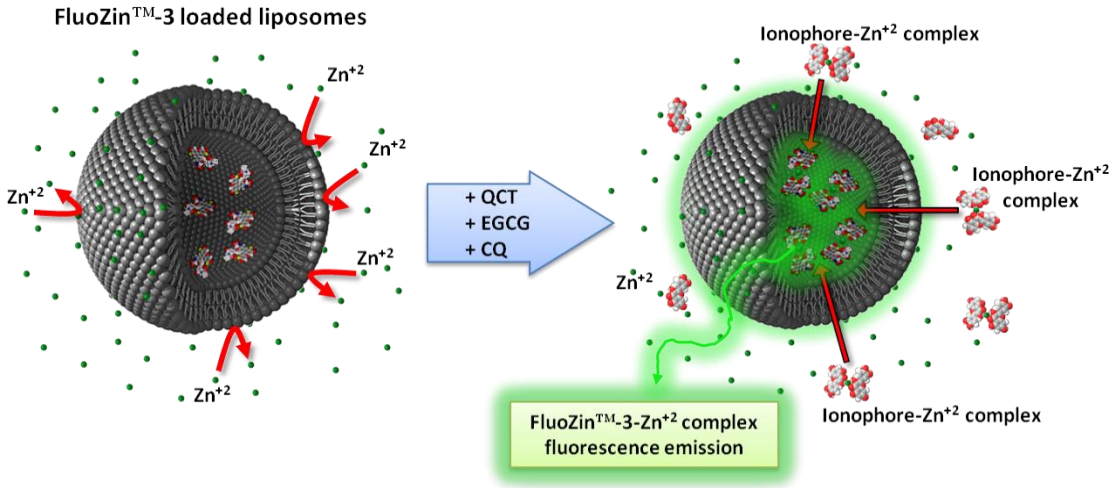

Figure 5.6. Schematic design of the FluoZin-3 loaded liposomes and the ionophore-like effect interpretation.

Following the addition of QCT, EGCG or CQ, the Zn-polyphenol chelation complex is formed and transported across the bilayer, followed by interaction with the encapsulated FluoZin-3 probe, resulting in a significant and immediate increase in the fluorescence intensity. Following the addition of 10 µM $ZnCl_2$ to the liposomes, a very small increase in the fluorescence appears due to the presence of a few free FluoZin-3 molecules that were not removed during the purification process and represent the background fluorescent signal. Even so, the fluorescent signal remains very low as zinc ions alone cannot cross the liposome membrane. The results from the kinetic experiment demonstrate that QCT, EGCG and CQ present different ionophore properties. CQ showed the strongest ionophore activity, producing a 35-fold increase in the zinc-dependent FluoZin-3 fluorescence intensity. Moreover, the maximum fluorescence is achieved rapidly after reaching the equilibrium in less than 15 min. QCT and EGCG also display a high ionophore activity in the system, although to a lesser extent as compared to the strong ionophore CQ, with 8- and 16-fold increases in fluorescence signal observed for QCT and EGCG, respectively. It can also be observed that both QCT and EGCG required more time (> 60 min) to achieve the plateau phase, displaying slower chelation and transport kinetics (Figure 5.7).

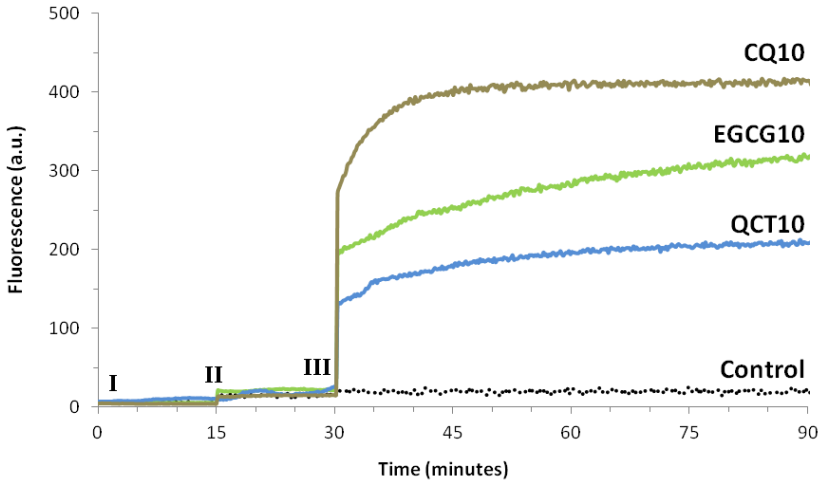

Figure 5.7. Effect of QCT, EGCG and CQ on the uptake of zinc cations by liposomes. Zinc-dependent fluorescence emission of FluoZin-3 encapsulated within liposomes treated with zinc cations, polyphenols, and CQ. The fluorescence emission (λ_{ex} = 494 nm; λ_{em} = 516 nm) of purified FluoZin-3-loaded liposomes was recorded continuously. Background fluorescence (0-15 min) was negligible (I). Upon the addition of 10 µM $ZnCl_2$ to the liposomal suspensions (II), a small fluorescence signal was detected, presumably due to the presence of trace amounts of unencapsulated FluoZin-3 in the liposomal solutions. At time point 30 min, 10 µM quercetin (QCT10), epigallocatechin-3-gallate (EGCG10), clioquinol (CQ10), or vehicle (control, final 0.1% DMSO) were added to the liposomal solutions, and the fluorescence was monitored for one additional hour (III).

Confocal microscopy analysis was also performed in order to visualize and corroborate that the fluorescence produced by the interaction between zinc and FluoZin-3 was attributable to fluorescence in the inner part of the liposomes. As can be seen in Figure 5.8, fluorescence is only observed when the combination of $ZnCl_2$ with QCT, EGCG or CQ is present, and the fluorescent signal comes from the inside part of the liposomes and not from the lipid membrane or the background solution. To further support this, stability studies were carried out in order to check whether the QCT, EGCG or CQ can destabilize and break the lipid vesicles. All the liposomes were characterized using TEM, DLS and ζ potential to check that their stability was maintained following exposure.

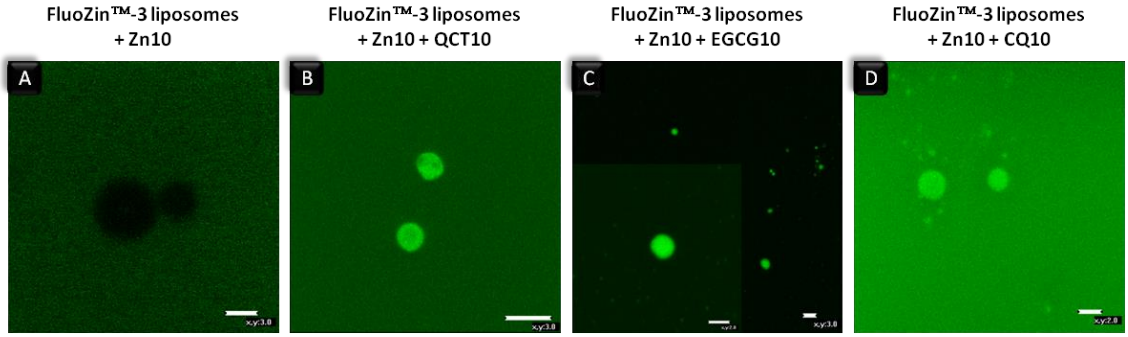

Figure 5.8. Three-dimensional confocal microscopy images of zinc dependent fluorescence emission of FluoZin-3 loaded within liposomes treated with zinc cations, polyphenols and CQ.

As shown in Figure 5.9, TEM images of the liposomes before and after treatments clearly demonstrate that liposome stability was not affected with the morphology and mean size (1-2 µm) being maintained. Moreover, the DLS and ζ potential results presented in Table 5.1, confirmed that the size of the liposomes and their surface charge were not significantly affected by the addition of zinc and/or the ionophores, thus demonstrating that FluoZin-3-loaded liposomes were not destabilized and their around-zero charge, due to the zwitterionic nature of main lipid component DPPC, was maintained, thus confirming that the fluorescence signal is due to transport of the Zn-QCT/EGCG/CQ complex across the lipid membrane.

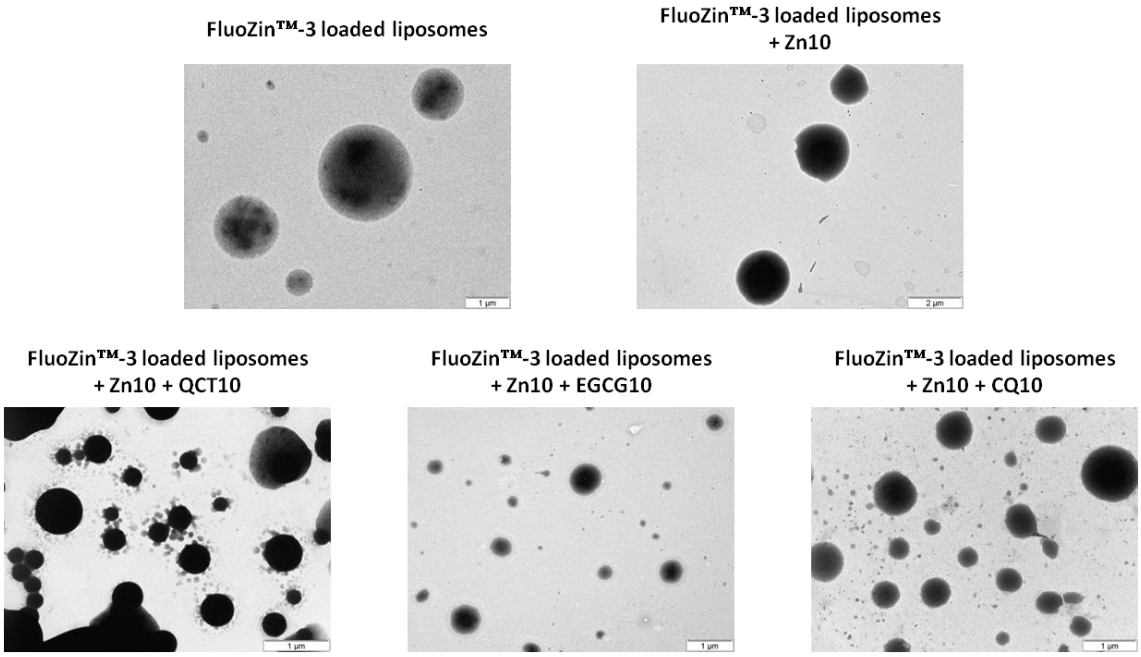

Figure 5.9. Transmission electron micrographs of liposomes with encapsulated FluoZin-3 after treatment with $ZnCl_2$, QCT, EGCG and CQ.

5.5. Discussion

The consequences that zinc chelation by flavonoids may have on zinc availability to the cells may in principle be dual: sequestering or ionophore, as shown for other well-characterized zinc-binding compounds.[45] On the one hand, the formation of zinc-flavonoid complexes may render zinc unavailable for cells, as do other dietary phytochemicals such as phytatesc[46] and zinc-

chelator drugs such as TPEN that induce zinc deficiency in vitro and in vivo.[47] Metal chelating therapy using CQ has been proposed for neurodegenerative disorders that course with high levels of metal accumulation such as Alzheimer's and Parkinson.[48] On the other hand, flavonoids may form water-insoluble membrane-permeant complexes with zinc that cross the plasma membrane, and thereby act as zinc ionophores. The ionophore effect of zinc binding compounds has been characterized for pyrithione and CQ. Both drugs chelate zinc cations and, when applied to cells, trigger a rapid increase of the intracellular pool of zinc that is detectable with different fluorophores such as Zinquin or FluoZin-3. Thereafter, it is assumed that these chelators form membrane-permeable complexes that are transported into the cell and that, once within the cell, chelator–zinc complexes dissociate into the single compounds due to the low concentration of intracellular free and labile zinc, thus providing labile zinc cations. Although FluoZin-3 has been widely accepted as a fluorophore that specifically interacts with zinc,[49–52] a recent report has indicated that this marker may in fact suffer from a lack of specificity.[53] However, this has no impact on the proof-of-concept study reported here as zinc is the only ion present and studied and thus no interfering effect from other ions will occur. We have shown here that treatment of Hepa 1-6 cells with zinc together with QCT, EGCG or CQ elicits a rapid and drastic increase in FluoZin-3-detectable intracellular zinc. The same effect was previously observed using Zinquin upon treatments of HepG2 cells with combinations of zinc and EGCG or zinc and QCT.[38] In these cells, the upregulation of MT and ZnT1 by zinc was enhanced by QCT. In contrast, EGCG decreased the intracellular zinc accumulation. Similar to QCT, the stilbene resveratrol efficiently chelates zinc in solution and enhances total and Zinquin-detectable cytoplasmic zinc in cultured human prostate epithelial cells, and this correlated with the antiproliferative action of resveratrol on cells.[33]

While an increasing effort has been made to understand the interaction of flavonoids with lipid bilayers,[54] no report has been published reporting the use of liposomes to demonstrate the zinc ionophore activity of polyphenols. Liposomes have been widely used as the simplest cell membrane systems in order to study the ionophore activity of molecules across the lipidic bilayer.[55] We have herein used a liposomal system to prove that zinc can transverse lipid bilayers when combined with flavonoids. It is not necessary to evoke the intervention of zinc transporters in the plasma membrane or the mobilization of zinc from intracellular compartments to account for the elevation of intracellular zinc levels in cells treated with flavonoids. The flavonoid-dependent transport of zinc cations into the liposomal cavity also implies that polyphenols may cross biological membranes when conjugated with metal cations. The mechanisms by which polyphenols enter the cells are largely unknown, but our results imply that complexation with metals may increase the bioavailability of polyphenols to cells.

There are several reports that strongly suggest that the demonstrated zinc ionophore effect on polyphenols will be observed in real physiological conditions if studied in vivo. Lee et al.[56] in 2002 has reported that a minor part of the EGCG found in plasma conserved its native form, and it has also been reported that the polyphenol metabolites still maintain their ability to chelate and form complexes with metal ions[30] and furthermore that nanomolar concentrations of polyphenols or their metabolites are able to modulate some metabolic pathways,[5,57,58] as does labile zinc in a picomolar to nanomolar concentration range.[14] While all these reports point toward the same ionophore effect being observed under physiological conditions, in a recent report by Oyama et al.,[59] the authors suggest the dual effect of CQ depending on the extracellular zinc concentration, where the known ionophore effect of CQ was only observed when extracellular zinc was available, and when zinc was not available in the extracellular environment, CQ could cross the membrane and chelate the intracellular zinc ions. To this end,

ongoing work is looking at extending the proof-of-concept reported and demonstrated here with both liposomes and cellular models to true physiological conditions exploring the interactions between a range of polyphenols and polyphenol metabolites and zinc in a lower concentration range of picomolar to micromolar.

In conclusion, we have demonstrated that QCT, EGCG and CQ rapidly increase intracellular labile zinc in Hepa 1-6 cells and that they function as ionophores for zinc in a liposomal system. Thus, natural flavonoids can be added to an arsenal of drugs that may be used to modulate zinc homeostasis and regulate zinc-dependent biological pathways.

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