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Anticancer properties of lipid and poly(ε-caprolactone) nanocapsules loaded with ferrocenyl tamoxifen derivatives

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Abstract

Objective: We synthesized new tamoxifen derivatives as anticancer drug candidates and elaborated on convection-enhanced delivery (CED) as strategy for delivery.

Methods: To overcome the issue of their poor solubility, these ferrocenyl-tamoxifen derivatives were esterified and encapsulated into different nanocarriers, i.e lipid (LNC) and polymeric nanocapsules (PNL-NC). We describe the chemistry, the encapsulation and the physicochemical characterization of these formulations.

Key findings: Starting compounds [phthalimido-ferrocidiphenol and succinimido-ferrocidiphenol], esterified prodrugs and their nanocapsules formulations were characterized. These drug candidates displayed a strong in vitro activity against breast and glioblastoma cancer cells. The ester prodrugs were toxic for glioblastoma cells (IC_{50} = 9.2x10^{-2} \mu\text{M} and 6.7x10^{-2} \mu\text{M respectively}). The IC_{50} values for breast cancer cells were higher for these compounds.

The encapsulation of the esterified compounds in LNCs (=50 nm) or PCL-NCs (=300 nm) did not prevent their efficacy on glioblastoma cells. These anticancer effects were due to both a blockade in the S-phase of the cell cycle and apoptosis. Moreover, the tamoxifen derivatives-loaded nanocapsules induced no toxicity for healthy astrocytes and showed no hemolytic properties. Loaded Lipid Nanocapsules (LNC) presented interesting profiles for the optimal delivery of active compounds.

Conclusion: Phthalimido- and Succinimido-esters represent an innovative approach to treat cancers with cerebral localizations such as glioblastoma or brain metastases from breast cancers.

Keywords (5): Lipid nanocapsules, polymer nanocapsules, ferrocenyl tamoxifen derivatives, breast cancer, glioblastoma.
1. Introduction

Tamoxifen has been used for endocrine therapy to treat breast cancer for many years. This selective estrogen receptor modulator (SERM) possesses an active metabolite named hydroxytamoxifen that competitively binds to estrogen receptor (ER) and thus inhibits cancer cell proliferation.\(^1\) Nonetheless, tamoxifen efficacy is only observed against estrogen receptor-positive tumors (ER\(^+\)), but resistance occurs after long-term usage.\(^2\) Importantly, tamoxifen is also able to treat other cancers, such as glioblastoma, in association with the standard of care, which is temolozomide.\(^3\) The embedding of a ferrocenyl unit into the tamoxifen skeleton can lead to a new family of breast cancer drug candidates named hydroxyferrocifens.\(^4,5\) Hydroxyferrocifens have the advantage of dual functionality. Effectively, these molecules have endocrine-modulating properties along with cytotoxic activities. A variety of hydroxyferrocifens were synthesized by structure-reactivity relationship studies.\(^6\) The activity remains when the dimethylaminoalkyl chain, which is inherited from the hydroxytamoxifen, is suppressed, leading to the ferrocidiphenol series. Among this series, some compounds with modification at the ethyl group, that is also inherited from hydroxytamoxifen, had better activity, in particular when a polar group was fixed at the end of the alkyl chain.\(^7,8\) Thus, the introduction of a polar imide group gave rise to ferrocidiphenol (Ferr) compounds, namely phthalimido-ferrocidiphenol (PhtFerr) (C\(_{35}\)H\(_{29}\)FeNO\(_4\)) and succinimido-ferrocidiphenol (SuccFerr) (C\(_{31}\)H\(_{29}\)FeNO\(_4\)), that were found to be very effective on breast cancer cells (MDA-MB-231) and, more surprisingly, on human glioblastoma cancer cells (U87).\(^9\) The mechanism underlying the Ferr cytotoxicity is only partly understood. Previous in vitro studies demonstrated that at least some Ferr metabolites produced in the cell are electrophilic quinonemethides that can induce growth arrest with
senescence and apoptosis phenomena.\textsuperscript{[10,11]} Recently, adducts of these quinonemethides with compounds bearing thiol functional group, as models of some cellular nucleophiles were identified.\textsuperscript{[12]} Metal-based anticancer drugs, such as PhtFerr and SuccFerr, are very hydrophobic compounds that cannot be formulated in aqueous solutions easily.\textsuperscript{[13]} Therefore, we both synthetized their respective ester prodrugs Phtester and Succester and also encapsulated them into nanocarriers to overcome the issue of their poor solubility. We compared two different formulations, \textit{i.e.} lipid nanocapsules (LNCs) and poly(\varepsilon\text{c}-caprolactone) nanocapsules (PCL-NCs), for encapsulation efficiency and release of the prodrugs of interest. These nanocapsules can be considered as platforms for a future development but, in a first step, they could be administered locally by Convection-Enhanced Delivery (CED).

LNCs (~100 nm) have a special structure between those of polymer nanocapsules and liposomes. These particles have various advantages, including a solvent-free manufacturing process and a prolonged physical stability of more than 18 months.\textsuperscript{[14]} Moreover, they allow the transport of multiple types of drugs, including lipophilic anticancer drugs such as paclitaxel, docetaxel, doxorubicin, hydroxytamoxifen, and etoposide;\textsuperscript{[15]} DNA and small interfering RNAs;\textsuperscript{[16,17]} radionuclides;\textsuperscript{[18,19]} and nuclease-resistant locked nucleic acids,\textsuperscript{[20]} offering a pharmaceutical solution for their parenteral administration.

PCL-NCs are larger vesicular systems (~300 nm) in which a lipophilic drug can be dissolved in an oily core and surrounded by a polymeric shell, allowing the drug to be absorbed onto the surface or entrapped within the nanocarriers.\textsuperscript{[21]} Some of the advantages of polymeric nanocapsules are their high loading capacity for lipophilic drugs, the protection they provide
against enzymatic degradation and their physicochemical stability. Moreover, PCL is a polyester polymer that is biodegradable and biocompatible.\(^{[22]}\) PCL is also considered a well-tolerated nanocarrier with very slow degradation.\(^{[23]}\) It was recently used as a safe scaffold for brain delivery of therapeutic agents.\(^{[24]}\) This paper details the physicochemical characterization of PhtFerr and SuccFerr and the synthesis of their respective esterified-derivatives Phtester and Succester. All of these compounds were loaded into LNCs and PCL-NCs. We studied the \textit{in vitro} drug release profile of the encapsulated prodrugs and their cytotoxicity against glioblastoma U87 cell lines and human breast MDA-MB-231 cancer (this latter cell line is known to be very sensitive to ferrocidiphenols). All these novel tamoxifen derivatives were more toxic for cancer cells compared to the parent drug tamoxifen, but less toxic for normal astrocytes. The strong and promising anticancer effect was demonstrated by both an efficient blockade of the cell cycle and also proapoptotic activity in breast and glioblastoma cancer cells.
2. Material and Methods

2.1. Chemistry

Reagents, molecules and chemical reactions are described in the supplementary material S1. The synthesis of PhtFerr and compound X have been previously described.[9] The complete chemical synthesis is detailed in S1. Measurements of the octanol/water partition coefficient \((\log P_{o/w})\) were made using high-performance liquid chromatography (HPLC) according to a method described previously.[25]

2.2. Solubility studies

Unless otherwise specified, all of the solvents were obtained from either Gattefossé (Nanterre, France) or Sigma Aldrich (Saint Quentin Fallavier, France).

The solubility of Phtester and Succester was determined as follows: two milliliters of each vehicle was added to screw-cap vials containing an excess of each of the Phtester and Succester compounds \((ie 500 \text{ mg})\). The mixture was heated in a shaking water bath (Memmert, Schwabach, Deutschland; \(25^\circ\text{C}, 48 \text{ h}, 60 \text{ strokes/min}\)) to improve the dissolution. When equilibrium was achieved, the mixture was centrifuged at \(1400 \times g\) for \(5 \text{ min}\), and the undissolved powder was discarded. Concentrations were determined by HPLC.

2.3. Preparation and loading of lipidic nanocapsules (LNC)

Phtester-LNCs and Succester-LNCs were prepared by a one-step process based on a phase-inversion temperature method described elsewhere.[26] To obtain LNCs, Solutol® HS15 (17% w/w), Lipoid® S75 (1.5% w/w, Lipoid Kosmetic, Grasse, France), Labrafac® (18.3% w/w),
Phtester or Succester (1.7%), NaCl (1.75% w/w) and water (59.75% w/w) were mixed and heated under magnetic stirring up to 85°C. Three cycles of progressive heating and cooling between 85°C and 60°C were then carried out and followed by an irreversible shock induced by dilution with 2°C deionized water (45 or 70% v/v) added to the mixture when it had reached 70–75°C. The resulting suspension was passed through a 0.2 µM filter to remove the free drug. The drug candidate load was expressed as the weight of drug in the lipid phase (in mg/g; after freeze-drying). The encapsulation yield (in %) was the amount of drug obtained at the end of the process divided by the initial amount of drug.

2.4. Preparation and loading of polymeric nanocapsules (PCL-NCs)

Phtester PCL-NCs and Succester PCL-NCs were prepared by a nanoprecipitation method that consisted of dissolving the molecules of interest (ie Phtester or Succester) in an organic phase: 1% triethyl citrate (with 5 mg/mL of PhtFerr or Succester), 10% alcoholic solution of lecithin (5 mg of lecithin/mL, Sigma Aldrich, Saint Quentin Fallavier, France) and 89% solution of PCL in acetone (1% of PCL, Sigma Aldrich, Saint Quentin Fallavier, France). This organic phase (10 mL) was added drop-wise under magnetic stirring to an aqueous solution of 10% Pluronic F68 (20 mL). Acetone and a portion of the water were then removed by evaporation in a vacuum at +40°C (Rotavapor Heidolf 94200) to reach a final volume of 15 mL. PCL-NCs were then purified on a gel column (ACA Ultroget® 54, Sigma Aldrich, Saint Quentin Fallavier, France). The drug candidate load was expressed as the weight of drug after freeze-drying (in mg/g of dried nanocapsules). The encapsulation yield (in %) was the amount of drug obtained after purification divided by the initial amount of drug.
2.5. Freeze-drying of nanocapsules

The samples were frozen in liquid nitrogen and freeze-dried (Labconco Freezone 6L). The temperature of each sample was equilibrated at -20°C for 72 h. After lyophilisation, the samples were stored at -20°C until further use.

2.6. Nanoparticles size and zeta potential

The size and zeta potential distribution of the nanocarriers were analyzed using a Malvern Zetasizer® Nano Series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK) operating at an angle of 90° and a temperature of 25°C (n=3). The nanocarriers were diluted 1:100 (v/v) in deionized water to ensure good scatter intensity on the detector. The zeta potential (n=3) was measured at pH 7.1 at 25°C at the same dilution using the following specifications: medium viscosity = 0.91 cP; refractive index (RI) = 1.33.

2.7. Drug payload in nanocarriers and encapsulation efficiency

Nanocarriers were first dissolved in acetonitrile. Then, the payload and encapsulation efficiency of LNCs and PCL-NCs were measured using an HPLC protocol. Twenty microliters of sample was injected into a C18 column (Nucleosil®, 5 µm, 0.46 mm, 25 cm; Macherey Nagel, Eckbolsheim, France) using an autosampler (Spectra Physics AS1000). The mobile phase was a mixture of acetonitrile and water (65:35, v/v) with a flow rate of 1.5 mL/min (Spectra Physics P1000XR; Thermo Electron S.A., Courtaboeuf, France). UV spectrophotometry was used to detect absorbance at 254 nm (Spectra Physics UV1000), and the peak area was used for quantification.

2.8. In vitro drug release from nanocarriers
The dialysis method was used as follows. Five milligrams of the lyophilized Phtester PCL-NCs, Succester PCL-NCs, Phtester LNCs, Succester LNCs, or Succester powder or Phtester powder were poured into 2 ml of 1% Tween® 20 and then transferred to a dialysis bag (molecular weight cutoff 14 kDa, Spectra/Por, Spectrum Laboratories, Paris, France). Each bag was placed into a bath of 498 mL of 1% Tween® 20 (pH 6.8). These preparations were placed in a bath shaking at 200 strokes/min at 25°C (Heito, France). One milliliter aliquots were taken at various intervals during the release period (24 h) and analyzed by HPLC to assess their drug content. The results are presented as the mean ± standard deviation (SD) of 3 experiments.

2.9. In vitro cytotoxicity

The cytotoxic activity of Phtester LNCs, Phtester PCL-NCs, Succester LNCs, Succester PCL-NCs, Phtester, Succester, PhtFerr and SuccFerr was assessed using healthy astrocytes, the U87 human glioblastoma cell line and the human breast cancer (MDA-MB-231) cell line. Purified newborn rat primary astrocytes were obtained by mechanical dissociation from cultures of cerebral cortex (authorization n° 2015.080410145453v3) as previously described.[27] Fisher male rats were obtained from Charles River Laboratories France (L’Arbresle, France).

The U87 and MDA-MB-231 cell lines were obtained from ATCC (Molsheim, France).

The cells were grown at 37 °C/5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen™, Life Technologies, Carlsbad, MA, USA) with glucose and L-glutamine (Cergy-Pontoise, France), 10% fetal calf serum (FCS) (BioWhittaker) and 1% antibiotic and antimycotic solution (Sigma, Saint-Quentin Fallavier, France). Phtester and Succester were dissolved in 1% dimethylsulfoxide (DMSO), whereas Phtester PCL-NCs, Phtester
LNCs, Succester LNCs, Succester PCL-NCs, blank LNCs and blank PCL-NCs were dispersed directly in DMEM. Tamoxifen was tested as a control drug.

All of the cells were seeded in sterile 96-well plates for 12 h with 100 µL of medium (5x10^3/well). Cells were then exposed to varying amounts of the indicated drug candidates at concentrations ranging from 10^-3 to 10^-9 M for 96 h at 37 °C. Blank nanocarriers were tested at an equivalent excipient concentration (compared to Phtester- or Succester-loaded PCL-NCs or LNCs). Cell cytotoxicity was then measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Ninety microliters of DMEM medium with 10% MTT was added to each well and incubated for 2-4 h at 37 °C. MTT was removed, followed by addition of solubilizing solution (100 µL DMSO), resulting in crystalline formazan formation. The absorbance was measured at 560 nm using a Beckman Coulter™ AD340S spectrophotometer. The absorbance is proportional to the number of viable cells, and survival was calculated as a percentage of the values measured for untreated cells.

2.10. Cell cycle analysis

In 6-well culture plates, 5 ×10^5 U87 cells were seeded in 6-well culture plates with 2 mL of medium per well. After 12 h, the culture medium was removed, and cells were treated with 10 µM of Phtester PCL-NCs, Phtester LNCs, Succester PCL-NCs, Succester LNCs, Phtester, Succester, PhtFerr, SuccFerr or blank formulations (PCL-NCs and LNCs); the culture plates were incubated for 96 h at 37 °C. At the end of the experiment, cells were trypsinized, washed twice with PBS (without Ca^{2+} and Mg^{2+}) and incubated with cold ethanol overnight at 4 °C. Cells were then centrifuged, washed once with PBS and then incubated with 0.5 mL PI/RNase (30 min, in the dark). The cell cycle was analyzed with an Accuri C6 flow cytometer (BD...
Biosciences) in at least three independent experiments, with 50,000 cells being measured in each sample, and data were analyzed by FCS Express 5 Software (De Novo Software, USA).

2.11. Cell apoptosis assay

The U87 cell line was incubated with 10 µM of different compounds (Phtester PCL-NCs, Phtester LNCs, Succester-PCL-NCs, Succester LNCs, Phtester, Succester, PhtFerr, or SuccFerr) for 96 h. Apoptotic cell death was revealed by flow cytometry using a phycoerythrin-conjugated monoclonal active caspase-3 antibody kit (BD Pharmingen, Le Pont de Claix, France) following the manufacturer’s instructions. The cells were analyzed with a BD-Accuri C6 flow cytometer, and the data were analyzed by FCS Express 5 Software. The percentage of apoptosis was obtained comparing it with the positive control.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Prism, La Jolla, USA). Kruskal–Wallis test (nonparametric) was used for cell cultures. Dunn’s post-test (nonparametric) was then used for multiple comparisons. Mann and Whitney test was used to compare the release of the complexes for each formulation.
3. Results

3.1. Chemistry

PhtFerr and SuccFerr were synthesized by substitution of the chlorine atom of 5-chloro-2-ferrocenyl-1,1-bis-(4-hydroxyphenyl)-pent-1-ene (compound X) with an imide (succinimide, Figure 1 and S1). The ester prodrugs (N-{4-ferrocenyl-5,5-bis-(4-acetoxyphenyl)-pent-4-enyl}phthalimide Phtester) and (N-{4-ferrocenyl-5,5-bis-(4-acetoxyphenyl)-pent-4-enyl}succinimide Succester) were obtained by acetylation of PhtFerr and SuccFerr, respectively (Figure 1). The characterization of the products is in supplementary materials S1.

3.2. Solubility studies

To find the optimal formulation of LNCs and PCL-NCs, we tested the dissolution properties of various surfactants [Solutol® HS 15 (polyoxyethylated 12-hydroxystearic acid), triethylcitrate, Capryol® 90 (propylene glycol monocaprylate), Transcutol® HP (highly purified diethylene glycol monoethyl ether), Labrasol® ALF (caprylocaproyl polyoxy-8 glycerides), Maisine® 35-1 (glycerol monolinoleate), Miglyol® 812 (caprylic/capric triglyceride, Dynamit Nobel, Leverkusen, Germany), Labrafac® (medium-chain triglycerides), Tween® 20 (polysorbate 20), Tween® 80 (polysorbate 80)] and oils [olive oil, benzylbenzoate, ethyl oleate, Captex® 355 (glyceryltributyrin/tricaprate), Triacetin (1,2,3-triacetoxypropane)].

The results of solubility studies are reported in Table 1. Among the surfactants, Solutol® HS15 and Labrafac®, usually used to make LNCs, have very good solubilization capacities.
Triethylcitrate, which has previously been used to make PCL-NCs, has quite similar properties. Finally, Tween®20 was used for solubility experiments. In each formulation (ie, LNCs or PCL-NCs), the ferrocenyl-tamoxifen derivatives were dissolved in oily droplets. Surfactants were used to increase the solubility of both Phtester and Succester.

3.3. Preparation and characterization of Phtester- and Succester-loaded nanocarriers

Because Labrafac® and Solutol® HS15 were included in the formulation of LNCs,[26] the dissolutions of the drug candidates were very easy. By mixing Phtester or Succester with excipients (ie, Labrafac®, Solutol® HS15 or Lipoid®) at well-characterized concentrations described by a ternary diagram[26] and by applying the phase-inversion process, Phtester LNCs and Succester LNCs were obtained. Their size ranges were very narrow with diameters between 53.4 ± 0.9 nm and 57.8 ± 2.0 nm (before lyophilisation), respectively, depending on the anticancer drug candidates payload (polydispersity index (PI) < 0.15) (Table 2). There was no significant difference before and after lyophilisation. Phtester LNCs and Succester LNCs were also characterized in terms of surface charge: zeta potential values were −10.8 ± 2.0 and −11.1 ± 1.3 mV, respectively. These physicochemical properties (Table 2) were very similar to those of the previously studied standard blank LNCs.[28,29] Indeed, because of the presence of PEG dipoles in their shells, 50-nm blank LNCs have a low zeta potential of approximately −10 mV.[30] The possible presence of the active drug on the surface did not affect the zeta potential values, which suggests that Phtester and Succester were efficiently encapsulated in the LNCs. Like many other hydrophobic drugs,[31-33] both compounds were mostly encapsulated in the LNCs with a high encapsulation yields above 92% (Table 2).
Phtester PCL-NCs and Succester PCL-NCs were obtained by nanoprecipitation. They had sizes of $303 \pm 12$ nm and $295 \pm 18$ nm, respectively, that were similar to the size of the blank ones ($289 \pm 7$ nm). The PCL-NCs loaded with Phtester and Succester exhibited negative charges of $-18.2 \pm 0.7$ mV and $-14.9 \pm 2.4$ mV, respectively. The incorporation of Phtester and Succester in PCL-NCs was very effective, as demonstrated by the high encapsulation yield and the zeta potential (Table 2).

### 3.4. Pharmaceutical properties

#### 3.4.1. *In vitro* release study

Phtester and Succester release studies were performed by a dialysis method. Under our conditions, the solubility of Phtester and Succester in the external compartment (Tween® 20, 1%) was 0.036 mg and 0.041 mg, respectively. The loaded LNC and PCL-NC formulations were both studied. Figure 2 illustrates the dissolution profiles obtained. Phtester PCL-NCs and Succester PCL-NCs were the most efficient formulations for effective drug release of greater than 70% after 12 h, whereas the diffusion from Phtester LNCs, Phtester, Succester-LNCs and Succester were quite slow, reaching only 30-40% release of the loaded drug candidates in the external phase after 24 h.
3.4.2. *In vitro* cytotoxicity on U87 cell lines

We performed MTT assays to evaluate the influence of the drug candidates on the viability of healthy astrocytes, MDA-MB-231 cells and U87 cells after treatment with Phtester- and Succester-loaded nanocapsules. A solution of 1% DMSO did not show any sign of toxicity. Therefore, the negative control was composed of the DMSO solution used to dissolve Phtester and Succester, and the corresponding viability was considered to be 100%. The blank formulations (nanocarriers alone) were non-toxic to breast cancer and glioblastoma cells and normal astrocytes in the micromolar range, as indicated in Table 3; the toxicity of tamoxifen was quite similar on the three cell lines.

PhtFerr and its prodrug Phtester were highly toxic to U87 cells, with very low IC$_{50}$ values of $1.6 \times 10^{-1}$ µM and $9.2 \times 10^{-2}$ µM, respectively. The IC$_{50}$ values for MDA-MB-231 cells were slightly higher for these compound but differences were not significant (Table 3; Kruskal Wallis, Dunn’s post hoc test vs U87: $p > 0.05$).

The IC$_{50}$ of PhtFerr and its prodrug Phtester were significantly higher (i.e. less toxic) on astrocytes (Table 3; Kruskal Wallis, Dunn’s post hoc test vs U87: $p < 0.05$). When encapsulated, the Phtester drug candidate conserved an important toxicity for glioblastoma cells.

Similarly, SuccFerr and its prodrug Succester were highly toxic to U87 cells, with very low IC$_{50}$ values of $2.7 \times 10^{-2}$ µM and $6.7 \times 10^{-2}$ µM, respectively (Table 3; Kruskal Wallis, Dunn’s post hoc test vs U87: $p < 0.05$). The encapsulation of Succester in LNCs or PCL-NCs did not alter or prevent the efficacy of the drug candidates on glioblastoma cells.

Importantly, Phtester- and Succester-loaded formulations did not alter cell viability for healthy astrocytes at the micromolar range.
3.4.3. Cell cycle analysis

The different treatments induced a major blockade in the subG1 and in S phases of the cell cycle (Figure 3) compared to their respective controls. This is in agreement with the cytotoxic activity of the different treatments (Table 3). Such an increase in the subG1 cell population revealed an alteration of the DNA content within the treated cells, which could occur either by necrosis or apoptosis.

3.4.4. Cell apoptosis assay

To investigate whether the native drugs candidates and their encapsulated forms induced apoptosis in U87 glioblastoma cells, we measured the induction of apoptosis with an active caspase-3 assay (Figure 4). All of the molecules and nanoformulations induced a significant increase in apoptosis after 96 h of treatment at 10 µM. We also observed a major pro-apoptotic effect of the PhTester PCL-NC formulation. All together, these results confirmed that the increased subG1 fractions observed (Figure 3) were due to the induction of apoptosis.
4. Discussion

Both PhtFerr and SuccFerr are tamoxifen derivatives and promising drug candidates for the treatment of cancer. However, these compounds are almost insoluble in water, and it is necessary to adapt their formulations to increase water solubility. Therefore, we decided to modify their chemistry by means of esterification and to encapsulate the resulting prodrugs Phtester and Succester into PCL-NCs and LNCs. Since we think that an intravenous administration of these nancapsules could induce hepatic toxicity, we proposed these formulations for a Convection-Enhanced Delivery (CED) for further in vivo experiments.

Polyester nanocapsules (PCL-NCs) can be produced by a solvent evaporation method: polymers and drug candidates are dissolved in an organic phase (ie oils and volatile water-miscible solvents) constituting the inner phase of an oil-in-water emulsion (O/W). The diffusion of the organic solvent into the aqueous phase causes precipitation of the polymer at the interface, and the final nanocarriers are made of an oily core and a polymeric shell. The blank PCL-NC formulation did not show any particular toxicity in our various cell models, as indicated by the very high IC$_{50}$ values on normal and cancer cells (Table 3).

In the present study, loaded LNCs and PCL-NCs had different drug release kinetics. Loaded PCL-NCs released approximately 60% of the initial drug amount within 6 h. This is considered very substantial compared to the release properties of LNCs, which showed a much slower release pattern (7 and 16%, respectively, for Phtester LNCs and Succester LNCs within 6 h). Consequently, both nanocarriers and their associated release patterns could be suitable for cancer treatment delivery, allowing fast or delayed delivery, respectively.

Considering drug-loaded nanocapsules, the mechanisms of cytotoxicity are a complex matter. The commonest method for lipophilic drugs to pass through cell membranes is passive
diffusion but if the drug is included in nanocarriers, the diffusion can occur only after the release in the medium (which is in favor of a lower toxicity); simultaneously, an adsorption of the nanocarriers on the surface can induce a direct toxicity or be followed by an uptake of the particles (phagocytosis, macropinocytosis, caveolae-mediated pathways, ...). Some of these mechanisms have been reported for U87 (eg phagocytosis[^34]) and for MDA-MB-231 (eg clathrin- and caveolae-mediated pathways[^35]). The accurate mechanisms of uptake have not been explored in this study; nevertheless, the in vitro experiments conducted with the nanocapsules loaded with hydroxyferrocifens ester prodrugs (ie Succester and Phtester) showed more cytotoxicity in cancer cells (MDA-MB-231 and U87) than in healthy astrocytes. This important result suggests that targeting either glioblastoma cells or brain metastases from breast cancer with encapsulated esterified hydroxyferrocifens could prevent off-target toxicity to normal surrounding healthy brain tissue.

The anticancer activity of these Ferr derivatives encapsulated into LNCs and PCL-NCs resulted from a combination of cytostatic effects (S-phase blockade) and cytotoxic effects (induction of apoptosis).

Importantly, LNCs have high drug-loading capacity, long-term physical stability, and a sustained drug release pattern, and they are able to enter the intracellular compartment of epithelial or glioma cells[^36]. For all of these reasons, it is widely accepted that LNCs represent one of the most promising nanoplatorms for central nervous system delivery[^36].
5. Conclusion

Taken together, our results indicate that the Phtester and Succester prodrugs are ferrocenyltamoxifen derivatives that are active against breast cancer and glioblastoma cells. Encapsulation of these molecules in LNCs does not modify their anticancer properties against these cell lines, though it does increase their solubility without adding toxicity to healthy astrocytes. We postulate that these formulations could be developed for CED administration and should be further explored for cancers located in the CNS, such as brain metastases of breast cancer or glioblastoma.
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Conflict of interest: The authors claim that no conflict of interest, financial or otherwise.
Table 1: Solubility of Phtester and Succester in various oils and surfactants

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<th>Solubility of Succester (mg/mL)</th>
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<td>Tween® 20 - 10%</td>
<td>16.7</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>Tween® 20 - 20%</td>
<td>16.7</td>
<td>43</td>
<td>29</td>
</tr>
<tr>
<td>Tween® 80</td>
<td>15</td>
<td>532 ± 8</td>
<td>619 ± 56</td>
</tr>
<tr>
<td>Solutol® HS 15</td>
<td>14-16</td>
<td>309 ± 61</td>
<td>408 ± 12</td>
</tr>
<tr>
<td>Labrasol® ALF</td>
<td>12</td>
<td>10.0 ± 0.1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Triethylcitrate</td>
<td>8.1</td>
<td>660 ± 17</td>
<td>432 ± 38</td>
</tr>
<tr>
<td>Capryol® 90</td>
<td>6</td>
<td>3.8 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Transcutol® HP</td>
<td>4.2</td>
<td>39 ± 0.1</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Maisine® 35-1</td>
<td>4</td>
<td>6.0x10^{-2} ± 0.1x10^{-2}</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Miglyol®812</td>
<td>15.4</td>
<td>7.2x10^{-4} ± 0.8x10^{-4}</td>
<td>6.1x10^{-4} ± 0.1x10^{-4}</td>
</tr>
<tr>
<td>Labrafac®</td>
<td>6</td>
<td>460 ± 5</td>
<td>465 ± 12</td>
</tr>
<tr>
<td>Benzoate benzyle</td>
<td>1</td>
<td>0.32 ± 0.01</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>Triacetin® Oil</td>
<td>5.7</td>
<td>5.7 ± 0.4</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>Ethyl oleate Oil</td>
<td>9.1x10^{-4} ± 0.6x10^{-4}</td>
<td>11.10^{-4} ± 1.10^{-4}</td>
<td></td>
</tr>
<tr>
<td>Captex® 355 Oil</td>
<td>9.3</td>
<td>9.3 ± 0.1</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>Olive oil Oil</td>
<td>6.1x10^{-2} ± 0.1x10^{-2}</td>
<td>0.5x10^{-3} ± 0.03x10^{-3}</td>
<td></td>
</tr>
</tbody>
</table>

* HLB: Hydrophilic Lipophilic Balance
Table 2: Mean characterization of the different nanocarriers formulations

<table>
<thead>
<tr>
<th></th>
<th>Mean particle size (nm)</th>
<th>Polydispersity index (PI)</th>
<th>Zeta potential (mV)</th>
<th>Drug load (mg/g)</th>
<th>Encapsulation yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank LNC</td>
<td>50.3 ± 0.2</td>
<td>0.071 ± 0.003</td>
<td>-8.9 ± 1.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phtester LNC</td>
<td>53.4 ± 0.9</td>
<td>0.134 ± 0.005</td>
<td>-10.8 ± 2.0</td>
<td>2.5 ± 0.6</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>Succester LNC</td>
<td>57.8 ± 2.0</td>
<td>0.118 ± 0.009</td>
<td>-11.1 ± 1.3</td>
<td>3.1 ± 0.2</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>Blank PCL-NC</td>
<td>289 ± 7.0</td>
<td>0.248 ± 0.011</td>
<td>-13.8 ± 0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phtester PCL-NC</td>
<td>303 ± 12</td>
<td>0.323 ± 0.003</td>
<td>-18.2 ± 0.7</td>
<td>9.0 ± 1.1</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>Succester PCL-NC</td>
<td>295 ± 18</td>
<td>0.267 ± 0.006</td>
<td>-14.9 ± 2.4</td>
<td>7.3 ± 0.4</td>
<td>81 ± 2</td>
</tr>
</tbody>
</table>

Measurements were made in triplicates (n=3) and results were expressed as mean values ± SD. Experimental drug load were expressed as the amount of drug in milligrams per gram of lipid nanocapsules suspension. Encapsulation efficiency was expressed as mean percentage (%) ± SD.
**Table 3**: Cytotoxicity (IC$_{50}$) of the indicated drugs on astrocytes, MDA-MB-231 and U87 cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Astrocytes</th>
<th>MDA-MB-231</th>
<th>U87</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank PCL-NC</td>
<td>7.4·10$^2$ [1.4·10$^2$-9.6·10$^1$]</td>
<td>1.8·10$^2$ [0.6·10$^2$-2.7·10$^1$]</td>
<td>4.0·10$^1$ [2.6·10$^1$-1.2·10$^1$]</td>
</tr>
<tr>
<td>Blank LNC</td>
<td>7.6·10$^2$ [1.9-2.2·10$^1$]</td>
<td>3.0·10$^1$ [6.3-2.3·10$^2$]</td>
<td>6.0·10$^1$ [8.8·10$^1$-1.2·10$^1$]</td>
</tr>
<tr>
<td>PhtFerr</td>
<td>3.7·10$^1$ [1.3·10$^1$-3.3·10$^2$]</td>
<td>2.0·10$^1$ [1.3·10$^1$-2.3·10$^2$]</td>
<td>1.6·10$^1$ [3.8·10$^1$-2.2·10$^1$]</td>
</tr>
<tr>
<td>Phtester</td>
<td>7.8·10$^2$ [1.0-7.1·10$^2$]</td>
<td>1.0 [0.28-5.6]</td>
<td>9.2·10$^2$ [3.8·10$^2$-2.2·10$^2$]</td>
</tr>
<tr>
<td>Phtester-LNC</td>
<td>8.0·10$^2$ [2.1·10$^2$-2.1·10$^1$]</td>
<td>2.1·10$^1$ [2.1-1.8·10$^2$]</td>
<td>7.0·10$^2$ [2.1·10$^2$-2.3·10$^1$]</td>
</tr>
<tr>
<td>Phtester PCL-NC</td>
<td>6.8·10$^2$ [1.1·10$^2$-7.3·10$^2$]</td>
<td>4.6·10$^1$ [5.1-2.4·10$^2$]</td>
<td>7.6·10$^2$ [2.1·10$^2$-2.3·10$^1$]</td>
</tr>
<tr>
<td>SuccFerr</td>
<td>3.4·10$^1$ [6.3-7.5·10$^1$]</td>
<td>3.3·10$^1$ [4.7·10$^1$-1.0]</td>
<td>2.7·10$^2$ [6.7·10$^1$-1.1·10$^1$]</td>
</tr>
<tr>
<td>Succester</td>
<td>7.1·10$^2$ [2.1·10$^2$-1.0·10$^3$]</td>
<td>6.0·10$^1$ [1.3·10$^1$-9.4·10$^1$]</td>
<td>6.7·10$^2$ [1.3·10$^2$-3.4·10$^2$]</td>
</tr>
<tr>
<td>Succester LNC</td>
<td>7.0·10$^2$ [1.2·10$^2$-8.1·10$^2$]</td>
<td>1.8·10$^1$ [1.5·10$^1$-1.4·10$^2$]</td>
<td>4.6·10$^2$ [1.5·10$^2$-1.4·10$^1$]</td>
</tr>
<tr>
<td>Succester-PCL-NC</td>
<td>7.8·10$^2$ [3.3·10$^2$-4.2·10$^3$]</td>
<td>2.0·10$^1$ [1.2·10$^1$-2.7·10$^1$]</td>
<td>5.7·10$^2$ [1.2·10$^2$-2.7·10$^1$]</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>1.85 [0.94-3.62]</td>
<td>2.63 [0.39-17.94]</td>
<td>1.92 [0.57-6.38]</td>
</tr>
</tbody>
</table>
References


Figure 1. Chemical structure and synthesis of the main compounds: Phthalimido-ester (Phtester) and Succinimido-ester (Succester) by acetylation of PhtFerr and SuccFerr. Synthesis of these precursors by substitution reaction on the chlorinated alkene X using phthalimide or succinimide.
Figure 2. Release profiles of a) Phtester [from Phtester (△), Phtester LNC (●), Phtester PCL-NC (■)] and b) Succester [from Succester (△), Succester LNC (●), and Succester PCL-NC (■)] by dialysis in Tween® 20 (1%) ; + different vs Phester, p < 0.05 (Mann and Whitney) ; * different vs Succester, p < 0.05 (Mann and Whitney).
Figure 3. Cell cycle distribution

![Cell cycle distribution diagram](image)

Figure 4. Apoptosis counting (panel a-b) and percentage of caspase-3 active cells (panel c) after 96 h of treatment

![Apoptosis graph](image)
Supplementary Materials for the Chemistry Section:

Chemical Reagents

The starting materials for the synthesis were acetic anhydride, pyridine, hydrochloric acid, sodium hydroxide, tetrahydrofuran (THF) magnesium sulfate, cyclohexane and ethyl acetate which were obtained from Sigma–Aldrich (L’Isle d’Abeau Chesnes, 38297 Saint-Quentin, Fallavier, France), TCI EUROPE N.V. (Boerenveldseweg 6, Haven 1063, 2070 Zwijndrecht, Belgique), and Alfa Aesar France (2 allée d’Oslo, 67300 Schiltigheim, France).

All reactions and manipulations were carried out under an argon atmosphere using standard Schlenk techniques. THF was distilled over sodium/benzophenone prior to use. Thin layer chromatography was performed on silica gel 60 GF254. IR spectra were obtained on a FT/IR-4100 JASCO 180 spectrometer. 1H and 13C NMR spectra were acquired on a Bruker 300 MHz spectrometer. Mass spectrometry was carried out at the “Service de Spectrométrie de Masse” at ENSCP, Paris. High-resolution mass spectra (HRMS) were acquired in the “Institut Parisien de Chimie Moléculaire (IPCM – UMR 8232)” at the “Université Pierre et Marie Curie”, Paris. Microanalyses were performed by the “Service de Microanalyse ICSN” at Gif sur Yvette, France. We already described the synthesis of PhtFerr compounds elsewhere (7).

Measurements of the octanol/water partition coefficient (log Po/w) were made by the HPLC technique according to a method described previously (see ref 21). Measurement of the chromatographic capacity factors (k) for each molecule was done at various concentrations in the range of 95–75% methanol containing 0.25% (v/v) 1-octanol and an aqueous phase consisting of 0.15% (v/v) n-decylamine in the buffering agent MOPS (3-morpholinopropane-1-sulfonic acid, prepared in 1-octanol saturated water) adjusted to pH 7.4. These capacity factors (k’) are extrapolated to 100% of the aqueous component given the value of k’w. The log Po/w is obtained by the formula log Po/w = 0.13418 + 0.98452 log k’.

The lipophilic Labrafac® CC (caprylic/capric acid triglycerides) was provided by Gattefosse S.A. (Saint-Priest, France). Lipoid® S75-3 (soybean lecithin at 69% of phosphatidylcholine) was a gift from Lipoid GmbH (Ludwigshafen, Germany); Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) was from Sigma-Aldrich (Saint Quentin Fallavier, France). Other reactants were obtained from Prolabo.
Deionised water was obtained from a Milli-Q plus system (Millipore, Paris, France). Poly(\(\varepsilon\)-caprolactone) (PCL), triethylcitrate and Pluronic (F 68 or F 127) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Egg lecithin was obtained from VWR (Fontenais-sous-Bois, France), Dulbecco modified Eagle medium (DMEM) with glucose and l-glutamine (Cergy-Pontoise, France), foetal calf serum (FCS) (BioWhittaker) and antibiotic and antimycotic solution (Sigma, Saint-Quentin Fallavier, France).

**Synthesis of SuccFerr:**

\(N\{4\text{-ferrocenyl-5,5\text{-bis\-(4-hydroxyphenyl\-pent-4\text{-enyl}\text{-succinimid}}e (SuccFerr):

A mixture of potassium carbonate (0.478 g, 3.5 mmoles) and succinimide (0.457 g, 4.6 mmoles) in dimethylformamide (DMF) was heated at 80°C for 15 min. The compound X (5-chloro-2-ferrocenyl-1,1-bis-(4-hydroxyphenyl)-pent-1-ene, 1.09 g, 2.31 mmoles) was added and the stirring was continued at 80°C overnight. The mixture was allowed to cool to room temperature, was poured into a diluted hydrochloric acid solution, was extracted twice with diethyl ether, then the organic layer was dried on magnesium sulfate and concentrated under reduced pressure. The residue was purified by flash-chromatography (ethyl acetate) to afford the imide SuccFerr that was obtained as an orange solid with a yield of 54% (0.662 g). mp: 225°C decomp. The corresponding NMR profile is described hereafter in the mass spectrometry section.

**Synthesis of Phtester:**

\(N\{4\text{-ferrocenyl-5,5\text{-bis\-(4-acetoxyphenyl\-pent-4\text{-enyl\-phthalimide (Phtester):}

Acetic anhydride (10 mL) was added dropwise to a solution of PhtFerr (3.01 g, 5.16 mmol) and pyridine (1.63 g, 1.7 mL, 20.6 mmol) in dry THF (50 mL) at RT then the reaction mixture was stirred at RT overnight. The solution was then poured into water (300 mL) in presence of hydrochloric acid (10 mL) and dichloromethane (300 mL) and the layers were separated. The aqueous layer was extracted twice with dichloromethane and the combined organic layers were washed with a solution of sodium hydroxide (2 g in 300 mL of water), then with water. The solution was dried over magnesium sulfate and concentrated under reduced pressure.
Flash chromatography (cyclohexane/ethyl acetate 1/1) then recrystallization from ethyl acetate yielded the pure product as an orange solid (2.87 g, 84%). Mp: 210°C. The corresponding NMR profile is described hereafter in the mass spectrometry section. The partition coefficient of the obtained Phtester was the following: Log Po/w: 6.09. This corresponds to a high hydrophobicity profile.

Synthesis of Succester:

N-{4-ferrocenyl-5,5-bis-(4-acetoxyphenyl)-pent-4-enyl}succinimide (Succester):

Acetic anhydride (18 mL) was added dropwise to a solution of SuccFerr (5 g, 9.34 mmol) and pyridine (2.95 g, 3.1 mL, 37.3 mmol) in dry THF (80 mL) at RT then the reaction mixture was stirred at RT overnight. The solution was then poured into water (400 mL) in addition to hydrochloric acid (15 mL) and dichloromethane (400 mL) and the layers were separated. The aqueous layer was extracted twice with dichloromethane and the combined organic layers were washed with a solution of sodium hydroxide (3 g in 400 mL of water), then with water. The solution was dried over magnesium sulfate and concentrated under reduced pressure. Flash chromatography (cyclohexane/ethyl acetate 1/1) then recrystallization from ethyl acetate yielded the pure product as an orange solid (5.3 g, 92%). Mp: 157°C. The corresponding NMR profile is described hereafter in the mass spectrometry section. The partition coefficient of the obtained Succester was the following: Log Po/w: 5.18. This corresponds to a high hydrophobicity profile.

Mass spectrometry: characterization of the products

N-{4-ferrocenyl-5,5-bis-(4-hydroxyphenyl)-pent-4-enyl}succinimide (SuccFerr):

$^1$H NMR (300 MHz, acetone-d$_6$) : δ 1.70-1.81 (m, 2H, CH$_2$), 2.56-2.64 (m, 6H, 2CH$_2$ succ+CH$_2$-C=C), 3.38 (t, $J = 6.6$ Hz, 2H, CH$_2$N), 3.95 (t, $J = 1.9$ Hz, 2H, C$_5$H$_5$), 4.10 (t, $J = 1.9$ Hz, 2H, C$_5$H$_5$), 4.16 (s, 5H, Cp), 6.74 (d, $J = 8.6$ Hz, 2H, C$_6$H$_4$), 6.85 (d, $J = 8.6$ Hz, 2H, C$_6$H$_4$), 7.04 (d, $J = 8.6$ Hz, 2H, C$_6$H$_4$), 8.25 (s, 1H, OH), 8.36 (s, 1H, OH). $^{13}$C NMR (75 MHz, acetone-d$_6$) : δ 29.4 (2CH$_2$, succinimide), 30.8 (CH$_2$), 33.8 (CH$_2$), 39.7 (CH$_2$), 69.5 (2CH, C$_5$H$_5$), 70.7 (5CH, Cp+2CH, C$_5$H$_5$), 88.8 (C, C$_5$H$_5$), 116.5 (2CH, C$_6$H$_4$), 116.6 (2CH, C$_6$H$_4$), 132.0 (2CH, C$_6$H$_4$), 132.4 (2CH, C$_6$H$_4$), 135.3 (C), 137.7 (C), 138.6 (C).
(C), 137.9 (C), 140.2 (C), 157.3 (C), 157.5 (C), 178.6 (2CO). IR (KBr, v cm⁻¹): 3421 (OH),
3096, 2967, 2936 (CH, CH₂), 1697 (CO). MS (ESI) m/z: 535 [M]⁺, 342, 279, 224, 143, 83.
C₃₁H₂₉FeNO₄(H₂O)₀.₅: C, 68.76; H, 5.14; N, 2.37.

N-{4-ferrocenyl-5,5-bis-(4-acetoxyphenyl)-pent-4-enyl}phthalimide (Phtester):

¹H NMR (DMSO-d₆): δ 1.70-1.89 (m, 2H, CH₂), 2.24 (s, 6H, Me), 3.50 (t, J = 6.4 Hz, 2H, CH₂N), 3.79 (t, J = 1.7 Hz, 2H, C₅H₄), 4.10 (s, 7H, Cp + C₅H₄), 6.89
(d, J = 8.5 Hz, 2H, C₆H₄), 7.02 (d, J = 8.8 Hz, 2H, C₆H₄), 7.06 (d, J = 8.8 Hz, 2H, C₆H₄), 7.16
(d, J = 8.5 Hz, 2H, C₆H₄), 7.86 (s, 4H, phthalimide). ¹³C NMR (DMSO-d₆): δ 20.8 (2CH₃),
29.3 (CH₂), 31.5 (CH₂), 37.3 (CH₂), 68.2 (2CH, C₅H₄), 68.7 (2CH, C₅H₄), 69.1 (5CH, Cp),
85.3 (C, C₅H₄), 121.4 (2CH, C₆H₄), 121.7 (2CH, C₆H₄), 122.9 (2CH, phthalimide), 129.5
(2CH, C₆H₄), 130.1 (2CH, C₆H₄), 131.4 (2C, phthalimide), 134.3 (2CH, phthalimide), 135.4
(C), 136.2 (C), 140.9 (C), 141.4 (C), 148.7 (C), 148.9 (C), 167.8 (2CO, phthalimide), 168.8
(COO), 169.0 (COO). IR (KBr, v cm⁻¹): 3454 (OH), 1766, 1752, 1708 (CO). HRMS (ESI,
C₃₉H₃₃FeNO₆: [M+Na]⁺) calcd: 690.154948, found: 690.15422.

N-{4-ferrocenyl-5,5-bis-(4-acetoxyphenyl)-pent-4-enyl}succinimide (Succester):

¹H NMR (acetone-d₆): δ 1.68-1.85 (m, 2H, CH₂), 2.23 (s, 3H, CH₃), 2.27 (s, 3H, CH₃), 2.50-
2.66 (m, 6H, 2CH₃succinimide + CH₂C=C), 3.37 (t, J = 6.5 Hz, 2H, CH₂N), 3.91 (s, 2H,
C₅H₄), 4.11 (s, 2H, C₅H₄), 4.15 (s, 5H, Cp), 7.01 (d, J = 8.2 Hz, 2H, C₆H₄), 7.11 (d, J = 8.2
Hz, 2H, C₆H₄), 7.12 (d, J = 8.2 Hz, 2H, C₆H₄), 7.26 (d, J = 8.2 Hz, 2H, C₆H₄). ¹³C NMR
(acetone-d₆): δ 21.00 (CH₃), 21.03 (CH₃), 28.7 (2CH₂, succinimide), 29.8 (CH₂), 33.1 (CH₂),
38.9 (CH₂), 69.2 (2CH, C₅H₄), 70.1 (5CH, Cp + 2CH, C₅H₄), 86.9 (C, C₅H₄), 122.5 (2CH,
C₅H₄), 122.6 (2CH, C₅H₄), 131.0 (2CH, C₅H₄), 131.4 (2CH, C₅H₄), 137.1 (C), 137.7 (C),
142.5 (C), 142.8 (C), 150.4 (C), 150.5 (C), 169.5 (COO), 169.7 (COO), 177.9 (2CO). IR
(KBr, v cm⁻¹): 3443 (OH), 1764, 1752, 1697 (CO). HRMS (ESI, C₃₅H₃₃FeNNaO₆: [M+Na]⁺)
calcd: 642.154948, found: 642.15387. Anal. Calcd for C₃₅H₃₃FeNO₆(H₂O)₀.₅: C, 66.88; H,
5.44; N, 2.23. Found: C, 67.26; H, 5.36; N, 2.09.