Magnesium complexes of ladanein: a beneficial strategy for stabilizing polyphenolic antivirals

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Abstract: Ladanein (noted FOMe) is a potent antiviral flavone that was shown to be active on a broad spectrum of enveloped viruses. This 5,6,7-trihydroxylated flavone has, however, pharmacokinetic properties and a half-life time that need to be improved for possible therapeutic applications. We herein took advantage of the complexation properties of ladanein (Fe(III)) to evaluate its ability to bind Mg(II) (biologically relevant and redox inert ion) precursors prepared beforehand from various carboxylic acids. The 5,6,7-trihydroxylated pattern of ladanein and the ligands borne by the Mg(II) atom of the precursors were found to be essential for firm Mg(II) binding. In particular, a ternary Mg(II) complex of ladanein and pidolate (noted FOMe.MgPid) was isolated and considered for its pharmacokinetic and virucidal (Hepatitis C Virus - HCV) properties. Mg(II) complexation significantly improved the physico-chemical (solubility) and the pharmacokinetic properties (clearance, plasmatic concentration) of the flavone FOMe, while not altering its anti-HCV capacity.

Introduction

Ladanein (extracted from dried leaves of *Marrubium Peregrinum L*) shares an uncommon 5,6,7-trihydroxylated substitution pattern on cycle A with several other flavones such as baicalein, oroxylin A, scutellarein or nornepetin (Figure 1). Most of these flavones were shown to display potent antiviral activities against viruses such as HCV, HIV, Influenza H1N1 or AMV viruses.[1] Ladanein was also shown to be effective against all major HCV genotypes, including a variant that is resistant to a reference entry inhibitor.[2] An interesting feature of these flavones is that most of their infectious targets are enveloped viruses and that, regardless of substitution on the B ring (Figure 1), the antiviral activity is maintained for a wide range of viral pathogens. For ladanein, a combined physico-(bio)-chemical approach suggested that iron(III) coordination of the β-hydroxy-ketone motif in A-C rings (Figure 1) is a key step in the bioactivation responsible for its observed anti-HCV activity.[3,4] The main drawbacks of ladanein, are, however, its low bioavailability and short half-life time (~7.3 minutes in mice serum). Even though it displays a pangenotypic activity, it is quickly metabolized in the body and hence its activity diminishes rapidly.

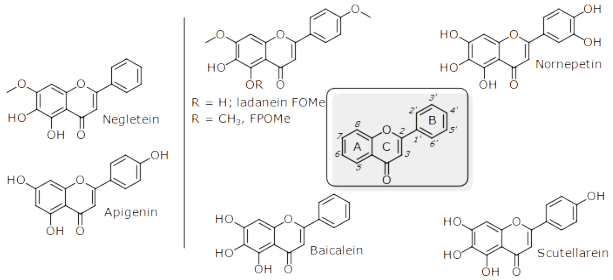


Figure 1. Chemical structures of 5,6,7-trihydroxylated flavones and analogous derivatives.

The low half-life time of ladanein was suggested to be due to degradation of the flavone *in vivo*, probably due to a 2-electron oxidation.[4] To protect the flavone from unspecific oxidation and to increase its bioavailability, complexation with magnesium(II) is herein investigated. Mg(II) is a biocompatible, biologically relevant and redox inert cation that is abundant in the human diet (whole grains, spinach, nuts or potatoes[5]). This second most abundant intracellular divalent ion (i.e., an adult human body contains ~24 g mainly present in bones (60 %) and muscles (27%)) plays an important role in more than 300 biological reactions (e.g., protein synthesis, reproduction, and DNA and RNA synthesis).[6] Mg(II) is also found in other cells or as extracellular magnesium.[7] Low magnesium levels may lead to e.g., hypocalcaemia,[8] osteoporosis[9] or hypertension.[10] Magnesium intake has beneficial effects for conditions such as migraine (magnesium citrate),[11] risk of stroke,[12] cardiovascular disease[13] or diabetes.[14] Different magnesium complexes are currently used as nutritional complements or as magnesium sources for drugs. Magnesium citrate (Figure 2) can be used in nephrolithiasis[15] (i.e., supplement because of its high bioavailability[16]), to treat leg cramps, either chronic[17] or pregnancy-induced[18] or for colon cleansing before a colonoscopy.[19] It is also used as a food preservative and is known as E345. Magnesium pidolate (Figure 2) is used in patients suffering from sickle cell disease.[20] Magnesium chloride (Figure 2) is used as a coagulant in the field of waste water

treatment[21] and to suppress dust and stabilize non-paved roads.[22] Magnesium sulfate (Figure 2) is widely used as a laxative,[23] to prevent eclampsia,[24] pre-term labour,[25] torsade de pointes[26] or in the treatment of acute asthma.[27] Finally, magnesium stearate (Figure 2) is used as an excipient and lubricant for medical tablets.[28] It is also used in aliments as a food additive and is known as E470b.

Although Mg(II) is widely used, the literature on flavonoid-magnesium complexes is extremely scarce. Only a few natural metalloanthocyanins (blue pigments containing Mg(II), e.g., commelinin from *Commelina communis*,[29] protodelphin from *Salvia Patens*,[30] protocyanin from *Centaurea cyanus*[31] or cyanosalvianin from *Salvia uliginosa*[32]) have been described. Within these pigments, intricate supramolecular organization between six anthocyanins and six flavones held together by six Mg(II) ions have been characterized. Each anthocyanin (under an anionic quinonoidal form) strongly interacts (π-π stacking) with a flavone (copigmentation), and Mg(II) was complexed both by the pyrogallol unit and the glucose borne by the B-ring of the flavone.

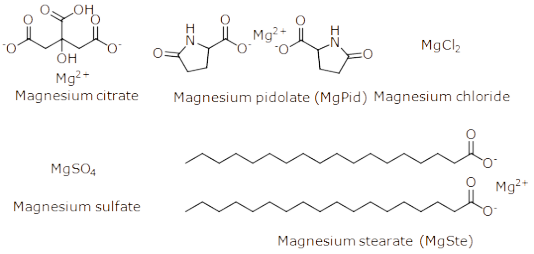


Figure 2. Various magnesium complexes.

The metal coordination ability of ladanein was already investigated with Fe(III).[3,4] Under physiological conditions, stable ternary flavone.FeNTA complexes (i.e., nitrilotriacetic acid NTA, used as an exogenous ligand, prevents formation of insoluble ferric species) were characterized and the exogenous NTA ligand was shown to prevent the flavone oxidation/degradation.[3,4] Following this work, the objective was herein to evaluate the possibility of complexing ladanein with Mg(II) and its effect on the stability, aqueous solubility and antiviral activity of the flavone (Figure 1).[33] The Mg(II) complexes have been characterized and quantified using UV-Vis. absorption spectrophotometry, ESI-MS, FTIR and cyclic voltammetry. Pharmacokinetic properties have been investigated and will be discussed in depth.

Results and Discussion

Mg(II) Coordination Properties of Ladanein with $MgCl_2$. A short, cheap and scalable synthesis of ladanein was recently developed.[33] The last step involved the deprotection of a methoxy group using $MgBr_2$ etherate in acetonitrile (CH_3CN). We therefore anticipated that the deprotection/complexing steps could be performed simultaneously in the presence of $MgCl_2$, thus constituting a smart formulation approach. The coordination of ladanein (FOMe) and $MgCl_2$ was first studied by absorption spectrophotometric means. Due to the low solubility of the $MgCl_2$ in CH_3CN, a mixed solvent made of 50% CH_3CN and 50% water (1:1 *v/v*) was first used to get as close as possible to the experimental reaction conditions, water being essential to ensure the solubility of $MgCl_2$. Figure 3a displays the absorption titration of ladanein FOMe by $MgCl_2$, and statistical analysis of these data allowed the determination of the stability constant of a FOMe.MgCl complex (log $K^*_{FOMe.MgCl}$ = 1.4(3)) as well as its spectral properties (Figure 3b). Ladanein is characterized by two intense absorption bands centred at 283 nm and 333 nm and assigned to π-π* transitions.[4] Coordination of Mg(II) by FOMe clearly induced a blending of these two absorption bands accompanied by the formation of an absorption tail of much lower intensity in the visible region (λ^{max} ~ 400 nm). Previous investigations also revealed the formation of a weak and broad absorption (λ^{max} ~ 425 nm) upon deprotonation of the 6-OH group of ladanein.[3,4] This long wavelength absorption measured for the monodeprotonated FOMe was attributed to a HOMO-LUMO transition (i.e., MO topology of the anion essentially unchanged from the neutral form with, however, a stronger contribution from the deprotonated oxygen in the HOMO). The nature of this Mg(II) complex of ladanein was further confirmed by electrospray mass spectrometry ESI-MS in the negative mode (Figure S1 in the ESI). Interestingly, $[FOMe + MgCl_2-H]^-$ and $[FOMe+MgCl-2H]^-$ species with *m/z* = 406.8 and 370.9, respectively, were detected and agreed with the calculated monoisotopic masses. Ionization of the Mg(II) complex is accomplished either by deprotonation or by addition of an anion (Cl^-). To ensure the importance of the 5- and/or 6-hydroxy groups in the complexation of Mg(II), an absorption titration of the final precursor FPOMe (the 5-methoxy analogue of ladanein, Figure 1) by $MgCl_2$ was performed under the same experimental conditions as above (Figure S2 in ESI). No spectral variation was observed for a [Mg(II)]/[FPOMe] ratio up to 1000 confirming the key role of 5,6-dihydroxyl group in the metal coordination.

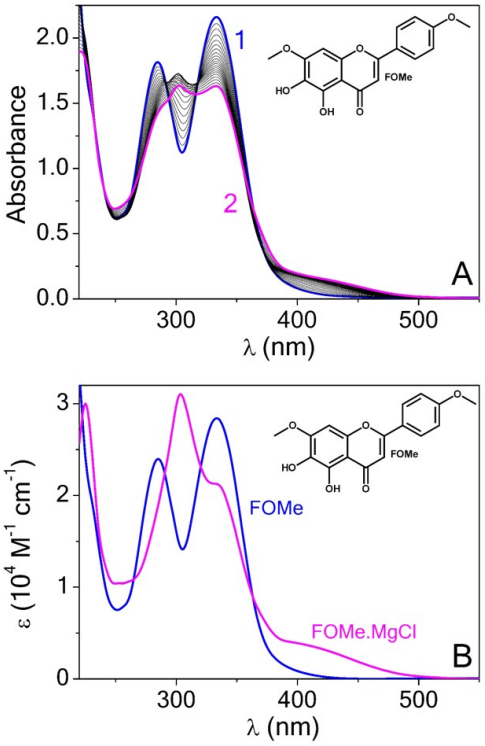


Figure 3. (A) UV-Vis. absorption spectrophotometric titration of FOMe by $MgCl_2$ and (B) absorption electronic spectra of the flavone FOMe and its magnesium(II) complex FOMe.MgCl. Solvent: CH_3CN/H_2O (1:1 *v/v*); *T* = 25.0 °C; *l* = 1 cm; $[FOMe]_{tot}$ = 7.63 × 10^{-5} M; (1) $[MgCl_2]_{tot}/[FOMe]_{tot}$ = 0; (2) $[MgCl_2]_{tot}/[FOMe]_{tot}$ = 185.8. The absorption spectra have not been corrected from dilution effects.

The low stability determined for FOMe.MgCl in CH_3CN/H_2O (1:1 *v/v*) however indicates that an excess of $MgCl_2$ (6 equivalents) would be required for the formation of more than 95% of the flavone-magnesium complex under the usual conditions used for demethylation (i.e., [FOMe] = 0.016 M, Figure S3 in the ESI). Deprotection of the FPOMe (0.06 M) by $MgCl_2$ (10 equivalents) was tested in CH_3CN at 80°C and absorption spectra were recorded on aliquots collected from the mixture at regular intervals for 40 hours. While deprotection of the 5-OCH_3 by $MgCl_2$ was found to be successful, the absorption spectra recorded over time did not show any evidence of concomitant complexation of Mg(II) by ladanein FOMe in CH_3CN. This demonstrates that, although possible, $MgCl_2$ is nevertheless far less reactive than $MgBr_2$ (i.e., deprotection is achieved after 40 hours of reaction with $MgCl_2$ instead of only 2 hours with $MgBr_2$) and that simultaneous a deprotection/complexing approach could not be used.

We next quantified the stability of the ladanein complex with $MgCl_2$ in pure methanol (Figure S4 in the ESI). Under these experimental conditions, the stability constant of FOMe.MgCl complex (log $K^*_{FOMe.MgCl}$ = 4.50(4)) was found to be more than 3 orders of magnitude higher than that measured in CH_3CN/water suggesting that water could compete with the binding and decrease the stability of the complex. To get a deeper insight, the complexation properties of ladanein were compared to other flavones of interest such as apigenin (i.e., lacking 6-OH group, Figure S5 in the ESI) and negletein (no substituent in the B-ring, Figures S6-S7 in the ESI) (Table 1 and Figure 1). Ladanein FOMe was found to display the highest affinity for Mg(II) in CH_3OH (log $K^*_{FOMe.MgCl}$ = 4.50(4)) with respect to apigenin (no complexation) or negletein FH (log $K^*_{FH.MgCl}$ = 1.97(4)). These data clearly evidenced the key role of the 5,6-dihydroxylation pattern on the A-ring as well as the electronic effect (resonance) of the B-ring substituent on the carbonyl unit.[3,4] Based on these data, we thus suggested the following structure for the Mg(II) complexes of ladanein FOMe (Figure 4).

Table 1. Stability constants of the different flavone-Mg(II) complexes (Solvent = CH_3OH; T = 25 °C; the errors are given as 3σ with σ = standard deviation).

	log $K^*_{F.MgX}$ (±3σ)			
F	$MgCl_2$	$Mg(Pid)_2$	$Mg(181)_2$	$Mg(Ste)_2$[a]
Ladanein FOMe	4.50(4)	4.92(7)	4.8(4)	4.1(6)
Negletein FH	1.97(4)	4.38(5)	4.77(8)	nd
Apigenin	nc	3.7(3)	4.12(4)	nd

nc = no complexation. nd = not determined. See Figure 1 for the chemical structures of ladanein, apigenin and negletein. [a] $Mg(Ste)_2$ dissolved in dioxane heated at 90°C and absorption titration performed in dioxane. X = Cl, Pid, 181 or Ste.

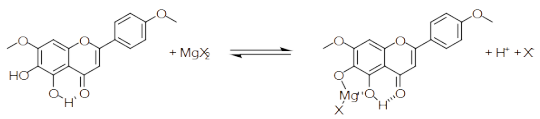


Figure 4. Proposed chemical structure of the Mg(II) complexes of ladanein FOMe.

Mg(II) Coordination Properties of Ladanein with Mg(II) Precursors. Following these preliminary results, the complexation properties of ladanein was then investigated in the presence of other Mg(II) precursors. Among the numerous salts safely employed in pharmacological compositions, magnesium(II) stearate (E572, noted $Mg(Ste)_2$) was first considered (Figure 2). Upon addition of Mg(II) stearate, the colour of the ladanein FOMe solution instantaneously changed from bright yellow (the colour of the free flavone) to dark orange, indicating the preferential formation of the complex. The complex has been isolated as an orange solid after recrystallization in CH_3CN. To confirm the formation of the flavone.MgSte complex, FTIR spectra of the free flavone, $Mg(Ste)_2$ and FOMe.MgSte were recorded. The corresponding spectra (Figure 5) clearly show the disappearance of the peak at 3518 cm^{-1} for FOMe.MgSte. Similarly to related compounds,[34] this absorption is likely due to the stretching of the free 6-OH group of ladanein (Figure 1; *i.e.,* the 5-OH group is involved in a strong hydrogen bonding with the C=O unit, Figure 4), which disappears when the complex is formed.[35] In addition, the C=O stretching band at 1676 cm^{-1} of the free flavone was found to be slightly shifted ($\Delta\upsilon$(C=O) by about 24 cm^{-1}) in the presence of $Mg(Ste)_2$ suggesting that the Mg(II) coordination has an impact on the ketone unit (Figure 5C).[36] No free stearic acid was observed since it has a very characteristic band around 1689 cm^{-1}, which is not present in the spectrum of the complexed flavone.

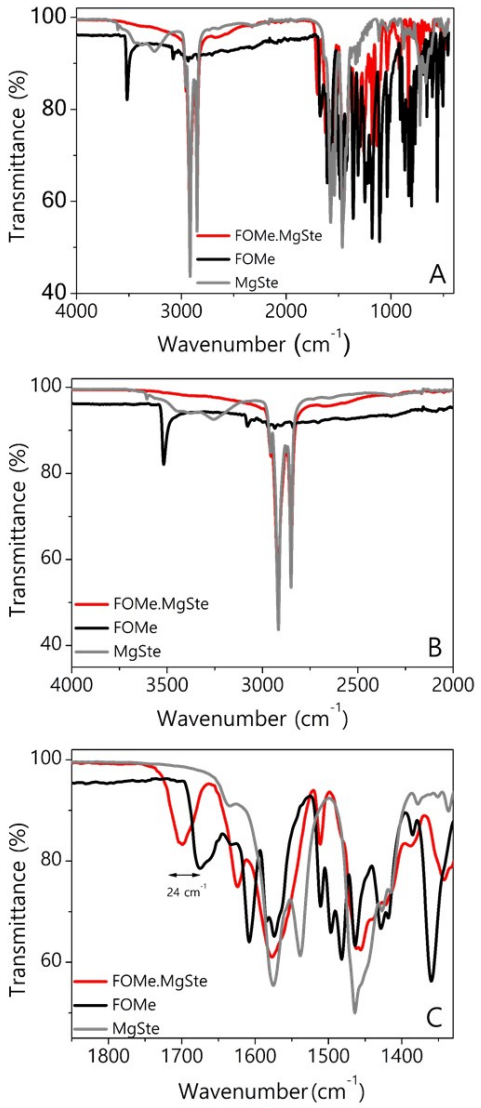
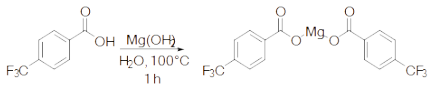


Figure 5. FTIR spectra of FOMe, $Mg(Ste)_2$ and FOMe.MgSte. (A) Whole spectrum; (B) zoom between 4000 and 2000 cm^{-1}; (C) zoom between 2000 and 1330 cm^{-1}.

To further evaluate the stability of the FOMe.MgSte complex, an absorption spectrophotometric titration was performed similarly to $MgCl_2$ (*vide supra*). However, $Mg(Ste)_2$ is only sparingly soluble in water, methanol or ethanol. A concentrated stock solution was therefore refluxed in dioxane and aliquots were added to a FOMe solution in a quartz optical cell. As previously observed for FOMe.MgCl (Figure 3), the formation of the complex can be monitored thanks to valuable spectral variations (Figure S8 in the ESI). Even though the experimental conditions were different, the stability of the FOMe.MgSte complex (log $K^*_{FOMe.MgSte}$ = 4.1(6)) is three orders of magnitude higher with respect to that of FOMe.MgCl (log $K^*_{FOMe.MgCl}$ = 1.4(3)) measured in CH_3CN/water (1:1 *v/v*) and comparable to that of FOMe.MgCl measured in CH_3OH (log $K^*_{FOMe.MgCl}$ = 4.50(4)). With respect to aqueous media, this stabilization is likely the result of the nature of the solvent as well as the auxiliary stearate ligands. Under the reaction conditions used the deprotection ([FOMe] = 0.016 M, *vide supra*, Figure S2 in the ESI), the addition of one equivalent would lead to the formation of more than 93% of the targeted magnesium complex (Figure S9 in the ESI).

Following these observations, we decided to investigate the synthesis of new Mg(II) precursors from commercial carboxylic acids. To optimize the reaction conditions, we first focused our attention on $Mg(Ste)_2$ that was prepared *via* a reported two-step pathway involving, first a deprotonation in the presence of NaOH followed by a reaction in the presence of $MgSO_4$. This sequence may be applied to other commercial carboxylic acids and could be used to form magnesium complexes containing the flavone and other functional molecules. This strategy was then applied to a commercial carboxylic acid (4-(trifluoromethyl)benzoic acid, noted 181) to afford the corresponding Mg(II) salt (noted $Mg(181)_2$). Nevertheless, the two-step synthesis used for the formation of magnesium(II) stearate failed to form the desired magnesium complex. Instead, a shorter synthetic route (Scheme 1) was developed involving the reaction between the desired carboxylic acid and magnesium(II) hydroxide, which acts both as the magnesium source and the base necessary for the deprotonation of the carboxylic acid. The Mg(II) precursor $Mg(181)_2$ was characterized by ESI-MS and ^{1}H NMR (Figure S10A and B in the ESI).



Scheme 1. Formation of the Mg(II) complex (noted Mg181) from a commercial carboxylic acid (noted 181).

A thorough physico-chemical investigation of the formation of Mg(II) complexes was then undertaken for ladanein FOMe and synthetic analogues (i.e., negletein and apigenin, Figure 1) using $Mg(Pid)_2$ and $Mg(181)_2$ as partners. Magnesium stearate ($Mg(Ste)_2$) was discarded because of its extremely low solubility in CH_3OH and the laborious procedure needed to perform the spectrophotometric titrations. Studies were performed using UV-Vis. absorption spectrophotometry followed by statistical analysis of the spectral data (ladanein, Figures S11-S14; negletein, Figures S15-S18 and apigenin, Figures S19-S20 in the ESI) in order to evaluate the stability constants of the respective ternary complexes (Table 1). For ladanein FOMe, the more stable complexes were found to be formed with $Mg(Pid)_2$ and $Mg(181)_2$ with log K* of 4.92(7) and 4.8(4), respectively. It is likely that the additional groups borne by the Mg(II) cation help stabilizing the ternary complexes compared to the chloride anion of $MgCl_2$. Furthermore, it can be also clearly seen that the complexes are by far much more stable in CH_3OH, probably because the water molecules compete with the binding and decrease the stability of the complex. Interestingly, this effect is amplified for FH, which forms much more stable complexes with $Mg(Pid)_2$ and $Mg(181)_2$ than $MgCl_2$ (Table 1). Participation of the β-hydroxy-ketone bidentate unit in the Mg(II) coordination could not be completely excluded since apigenin was also found to complex both $Mg(Pid)_2$ and $Mg(181)_2$ in CH_3OH (Figures S19-S20 in the ESI) but to a lesser extent compared to FOMe. No complexation of $MgCl_2$ could be observed in CH_3OH confirming the essential role of auxiliary ligands for Mg(II) complexes.

In order to corroborate the formation of the magnesium complexes with FOMe and its analogues, an ESI-MS study was undertaken. Solutions of the flavones and Mg(II) precursors at 10^{-4} M were mixed immediately before injection and the outcome was observed. All complexes were clearly characterized. Their isotopic profiles showed an excellent agreement with the simulated ones thus substantiating our observations. The formation of the FOMe.MgPid complex was also assessed by ^{1}H NMR spectroscopy. FOMe.MgPid was prepared by mixing stoichiometric amounts of FOMe and $Mg(Pid)_2$ (reflux at 80°C in CH_3CN overnight) to yield a light green solid that was recrystallized from CH_3CN. The comparison of the ^{1}H (Figure 6) and ^{13}C (Figures S21-S23 in the ESI) NMR data recorded for FOMe.MgPid, $Mg(Pid)_2$ and FOMe in DMSO-d_6 provided interesting information (Figure 6). FOMe is characterized by two singlet signals at 12.60 and 8.71 ppm that were attributed to the 5- and 6-OH, respectively. Upon complexation with Mg(II), the 6-OH completely disappeared while that attributed to 5-OH could still be observed as a broadened signal suggesting its involvement in the Mg(II) coordination. Furthermore, the signals corresponding to the methoxy groups and aromatic protons were slightly shifted when compared to the free flavone. In addition, the N*H* proton of the pidolate moiety was also affected by Mg(II) complexation. Finally, although the proton signals measured for FOMe.MgPid are broadened, these data suggest the involvement of a FOMe flavone molecule and a single pidolate within the Mg(II) complex reinforcing our initial hypothesis regarding the possible structure of the complex.

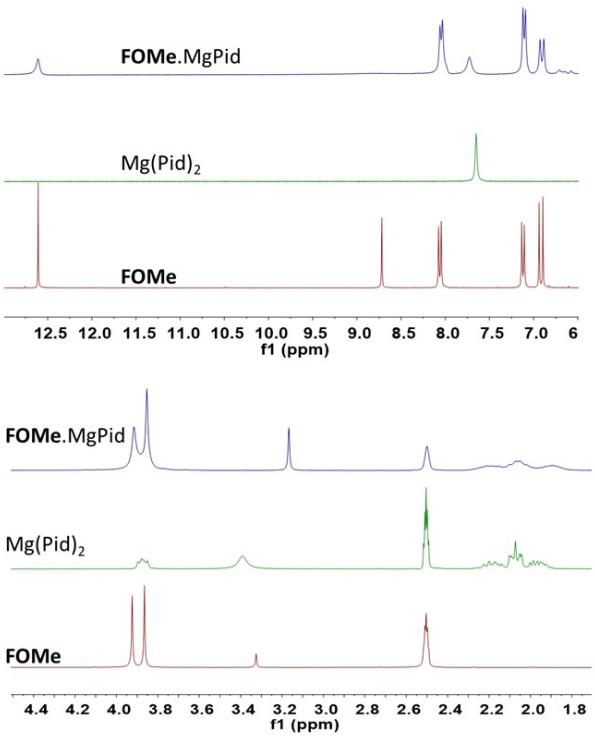


Figure 6. ^{1}H NMR spectra of FOMe compared to its isolated magnesium ternary complex FOMe.MgPid and the commercially available $Mg(Pid)_2$. Solvent: DMSO-d_6

Comparison of the ^{13}C NMR data of the ternary complex with $Mg(Pid)_2$ and FOMe shows in general a significant reduction of the intensity and a broadening of the signals of the A and C rings of the ladanein FOMe (Figure 1) confirming the involvement of these units in the complexation of Mg(II). Furthermore, marked differences were observed for the ^{13}C NMR signals of the pidolate partner. One of the C=O signals involved in the coordination of Mg(II) at 178 ppm completely disappeared while upfield shifts of 0.3-0.4 ppm of the ^{13}C signals close to the carboxylate unit (CH and CH_2) were observed. All these data corroborate the observations made by ^{1}H NMR and the nature of the proposed ternary Mg(II) complex.

Pharmacokinetic properties of Ladanein and of its Mg(II) complex with Pidolate. Thermodynamic solubilities of the free flavone FOMe and its FOMe.MgPid complex were first measured in an aqueous pH 7.4 PBS buffer at 21 °C. The solubility of the Mg(II) complex was measured to be 3.7-fold higher than the free flavone (Table 2). This finding is consistent with the pharmacokinetic analysis of the bioavailability data (Table 2) obtained in tests using groups of 12 CD-1 mice treated both orally (*per os*, PO) and intravenously (IV). The area under the curve (AUC) is generally calculated from the time of administration until the concentration of the drug is negligible and represents the total exposure to the drug over time. This value is usually employed to define the maximal plasmatic concentration over time. Interestingly, both the AUC_{IV} and AUC_{PO} values of FOMe.MgPid complex were found 5.8-fold and 3.4-fold higher than those of the free flavone FOMe, respectively, following administration of equimolar doses. Furthermore, the clearance (Cl), defined as the rate at which waste substances are cleared from the blood, either via metabolism or via elimination in the urine, is also decreased by 3-fold for FOMe.MgPid complex with respect to FOMe (Table 2). These pharmacokinetic parameters measured for FOMe and its Mg(II) complex, FOMe.MgPid (Table 2) thus demonstrate a better stability of the flavone upon Mg(II) complexation.

Table 2. Pharmacokinetic properties of the FOMe.MgPid complex and FOMe in CD-1 mice.

	FOMe	FOMe.MgPid
Solubility in PBS pH 7.4 (µM)[a]	23 ± 7	86 ± 35
$t_{1/2}$ (min)	7 ± 1	8 ± 1
Cl (mL/min/kg)	442 ± 116	143 ± 20
AUC last IV (min.ng/mL)[b]	566 ± 134	3295 ± 457
AUC last PO (min.ng/mL)[c]	171 ± 27	575 ± 70

[a] Measured at pH 7.4, PBS buffer-, 24 h of incubation, 20°C; [b] IV: drug administered intravenously; [b]PO (per os): administration of the drug done orally.

At a first glance, the biological half-life times measured for ladanein FOMe and FOMe.MgPid (Table 2) can be considered as low. However, they are comparable to other compounds. For example, midazolam, a drug used for the treatment of insomnia or seizures has an *in vivo* half-life time in the mouse of ~ 12 minutes. Other compounds such as antipyrine or caffeine display higher half-life times of 28 and 30 minutes, respectively. Noteworthy, these three compounds showed much higher half-life times when tested on humans (*i.e.,* in humans, $t_{1/2}$ = 4, 12 and 5 hours for midazolam, antipyrine and caffeine, respectively). It is thus expected that $t_{1/2}$ values of both ladanein and its magnesium pidolate complex could be significantly improved if tested on humans. When compared to other polyphenols with reported anti-HCV activities (e.g., epigallocatechin gallate EGCG with $t_{1/2}$ of ~ 200 minutes in mice), FOMe and FOMe.MgPid were found to display a much lower $t_{1/2}$ value.[37]

Antiviral Activity of FOMe.MgPid. The antiviral tests were then only performed with FOMe.MgPid, which has proven to be the most interesting Mg(II) complex in terms of stability and solubility. The antiviral activity of FOMe.MgPid was evaluated to verify whether it is retained when the flavone is complexed with Mg(II). Interestingly, the Mg(II) pidolate complex of ladanein FOMe showed similar anti-HCV potency (Figure 7) with respect to free FOMe. The EC_{50} was calculated for each compound and was found to be 1.6 ± 0.2 µM for ladanein FOMe and 1.5 ± 0.1 µM for FOMe.MgPid, respectively. Thus, it can be observed that the combination with a Mg(II) precursor has no or little effect on the antiviral activity of ladanein FOMe *in vitro*, while it significantly improves the pharmacokinetic properties of the flavone.

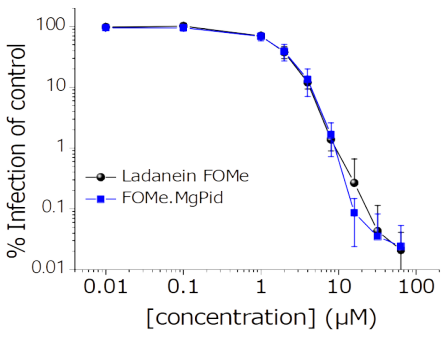


Figure 7. Anti-HCV activities of ladanein and its magnesium pidolate complex. Summary of four independent experiments. HCV Jc-R2a reporter virus was pre-incubated (20 min to 1h) with indicated concentration of drugs at 37 °C

prior to infection of Huh-7.5 cells. Cells were subsequently lysed 72 h post infection and luciferase activity was then measured.

Conclusion

The Mg(II) complexes of the antiviral ladanein flavone FOMe and related analogues (negletein FH and apigenin) were studied by spectrophotometric and mass spectrometric means. We clearly demonstrated the Mg(II) binding ability of ladanein thanks to its substitution pattern on cycle A (5,6,7-trihydroxylated). The 6-OH group has been shown to be a key element in the Mg(II) coordination, the coordination sphere of Mg(II) being completed by an auxiliary ligand and the contribution of the 5-OH unit of the flavone. The stability of these complexes was found to be closely dependent on the ligand borne by the Mg(II) atom of the precursors. One of the complexes, FOMe.MgPid, was prepared and its antiviral activity and pharmacokinetic properties were evaluated. The Mg(II) complex was shown to display the same antiviral activity as ladanein FOMe with a better solubility and lower clearance. Furthermore, the plasmatic concentrations of FOMe and FOMe.MgPid were superior to their EC_{50} values. Using this strategy, we have shown that carboxylic acid derivatives can be used to obtain ternary complexes of Mg(II) with an antiviral flavone allowing better control of the stability, solubility, and electronic and steric characteristics of the target species. Efforts are currently ongoing to combine two antiviral molecules in the same entity through a Mg(II) link.

Experimental Section

Starting Materials and Solvents. Ladanein FOMe and negletein FH were synthesized according a previously described procedure.[33] Magnesium(II) chloride ($MgCl_2$, anhydrous >98 %), magnesium(II) stearate ($Mg(C_{18}H_{35}O_2)_2$, puriss.), magnesium(II) pidolate ($Mg(C_5H_6NO_3)_2$ 98 %), magnesium(II) hydroxide ($Mg(OH)_2$, > 99%), 4-(trifluoromethyl)benzoic acid ($C_8H_5O_2F_3$, 98%) and apigenin ($C_{15}H_{10}O_5$, >97%) were purchased from Sigma Aldrich and used without further purification. Distilled water was further purified by passing it through a mixed bed of ion-exchanger (Bioblock Scientific R3-83002, M3-83006) and activated carbon (Bioblock Scientific ORC-83005) and was de-oxygenated by CO_2- and O_2-free argon (Sigma Oxiclear cartridge) before use. Spectrophotometric grade methanol (Merck, p.a.) was de-oxygenated by CO_2- and O_2-free argon (Sigma Oxiclear cartridge). All the stock solutions were prepared by weighing solid products using an AG 245 Mettler Toledo analytical balance (precision 0.01 mg). All measurements were carried out at 25.0(2) °C.

Infrared Spectra. Infrared (IR) spectra were recorded neat on a Perkin-Elmer Spectrum One Spectrophotometer.

Synthesis of FOMe.MgPid. Ladanein FOMe (152 mg, 0.48 mmol) is mixed with 136 mg $Mg(Pid)_2$ in 40 mL CH_3CN under argon. The mixture is heated to 80 °C for 16 hours. The solution is then cooled to room temperature and the solvent is partially removed under reduced pressure. A light green solid precipitates and is then filtered and dried to quantitatively afford the target FOMe.MgPid complex. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) 12.60 (br s), 8.03 (d, *J* = 11.2 Hz, 2H, ladanein, B ring), 7.72 (s, 1H, pidolate NH), 7.11 (d, *J* = 11.2 Hz, 2H, ladanein, B ring), 6.92 (s, 1H, ladanein, A ring), 6.92 (s, 1H, ladanein, C ring), 4.0 – 3.7 (m, 7H, Ladanein OCH_3 and CH pidolate), 2.35 – 1.7 (m, 4H, CH_2 pidolate). ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) 182.25, 177.11, 163.34, 162.30, 154.44, 149.68, 146.19, 130.01, 128.25, 123.02, 114.59, 105.11, 103.18, 91.23, 56.33, 55.59, 55.53, 29.39, 25.09.

Spectrophotometric Titrations of the Flavones by Mg(II). Stock solutions of flavone (from 3.07×10^{-3} M to 7.60×10^{-4} M) were prepared in methanol and then further diluted with either methanol or a mixed solvent made of CH_3CN and water (*v/v*) to obtain a ligand concentration ranging from 7.55×10^{-5} M to 1.32×10^{-5} M. The spectrophotometric titrations of the flavones by the various Mg(II) precursors were thus carried out in CH_3OH or CH_3CN:H_2O (*v/v*). Microvolumes of a concentrated solution of Mg(II) complexes (from 1.27×10^{-2} M to 6.17×10^{-3} M) were added to 2 mL of the ligand solutions in a 1 cm path length quartz optical cell. When studying the complexation of $Mg(Ste)_2$ by ladanein FOMe, the poor solubility of the Mg(II) precursor required heating the stock solution of $Mg(Ste)_2$ in dioxane to reflux at 90°C and adding microvolumes of it to 2 mL of a ladanein solution contained in a 1 cm path length quartz suprasil cell. During the titrations, UV-Vis. absorption spectra were recorded from 200 nm to 800 nm on a Cary 5000 (Agilent) or Cary 50 (Varian) spectrophotometer maintained at 25.0(2) °C by the flow of a Cary Varian Dual Cell Peltier accessory or a Lauda E200 thermostat, respectively.

Analysis and Processing of the Spectroscopic Data. The spectrophotometric data were analysed with Specfit program[38] which adjusts the absorptivities and the stability constants of the species formed at equilibrium. Specfit uses factor analysis to reduce the absorbance matrix and to extract the eigenvalues prior to the multiwavelength fit of the reduced data set according to the Marquardt algorithm.[39]

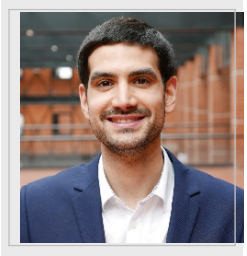
Electrospray Mass Spectrometric Measurements. Electrospray mass spectra of metal complexes of the different flavones were obtained with an AGILENT TECHNOLOGIES 6120 quadripole equipped with an electrospray (ESI) interface. Solutions (~10^{-4} M) of the Mg(II) complexes of the flavones were prepared in a methanol:water (80:20 *w/w*). The sample solutions were continuously introduced into the spectrometer source with a syringe pump (KD SCIENTIFIC) at a flow rate of 800 µL/h. For electrospray ionization, the drying gas was heated at 250 °C and its flow was set at 6 L.min^{-1}. The quadrupole temperature was set to 100 °C. The capillary exit voltage was fixed at 5 kV and the skimmer voltage was varied from 100 to 250 V in negative mode in order to optimize the signal responses. Scanning was performed from m/z = 100 to 1000.

Physicochemical and Pharmacokinetics Studies. Solubility and bioavailability studies were performed by Patrick Gizzi, PCBIS (UMS CNRS 3286, ESBS-Université de Strasbourg, Illkirch, France). Physicochemical studies were performed on a Gilson HPLC system with a photodiode array detector, an autosampler and a Valco injector. Data acquisition and processing were performed with Trilution LC V2.0 software. Measurements were carried out at room temperature. A 2.6 µm kinetex C18 100 A (50 X 4.6 mm) column, purchased from Phenomenex, was used for solubility studies. Thermodynamic solubilities of free ladanein FOMe and of its FOMe.MgPid complex were measured by dissolving the compounds until saturation in PBS pH 7.4 buffer. Samples were shaken during 24 hours at 21 °C. Saturation was confirmed by the presence of undissolved powder. After ultracentrifugation, a HPLC procedure by diluting the 10 mM DMSO stock solution to adapted concentrations in acetonitrile/water solution and using a calibration line established for the free flavone FOMe and its FOMe.MgPid complex measured the concentration in the supernatant. The injection volume was 20 µL for both derivatives, the mobile phase flow rate was 2 mL/min and the following program was applied for the elution: 0-0.1 min, 5% B; 2.6-3.1 min, 5-95% B; 3.3-6 min, 5% B. Solvent A was an aqueous solution containing 0.05% trifluoroacetic acid and solvent B was HPLC grade acetonitrile (Sigma-Aldrich CHROMASOLV). The detection wavelength was 330 nm and the retention times were the following: FOMe, 2.01 min; FOMe.MgPid, 2.03 min.

IV and Oral Bioavailability. FOMe or FOMe.MgPid solutions were prepared in a physiological serum containing 10% cremophor. CD-1 mice (20–25 g, twelve per group) were given a single equimolar dose (0.79 µmol/kg) of FOMe or FOMe.MgPid by IV route (2 µL/g) or by oral route (4x diluted in water, 8 µL/g). The mice were then sacrificed at 5, 15, 30, 60, 120 and 180 min, Plasma were collected and frozen at -80 °C until they are processed as followed. Each plasma sample (400 µL) was then mixed with acetonitrile (1 mL) to trigger protein precipitation and to extract the compounds. The samples were stirred using a vortex and

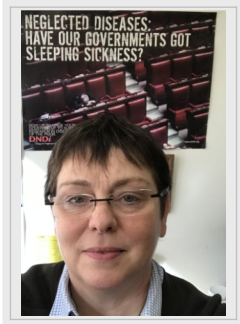
sonicated for 3 min. The precipitated proteins were centrifuged for 5 min at 4 °C (15 000g). The supernatants were transferred in a microtiter plate to be analysed by LC-MS/MS (UHPLC coupled to triple quadripole Shimadzu LC-MS 8030). A calibration curve was prepared by preparing known varying compound concentrations in plasma and analysing by the same method. Compounds in plasma were identified by comparing samples with the retention times of authentic standards. Quantification was based on comparison of peak heights with standard plasma containing a known amount of compound. All animal experiments were performed in accordance with institutional guidelines and approved by the French Ministère de l'Enseignement Supérieur de la Recherche et de l'Innovation and local ethical committee (CREMEAS) according the APAFiS procedure (agreement number #3670-201601201121159).

Antiviral Activity. The antiviral activities of FOMe and FOMe.MgPid have been determined as described elsewhere.[2] The Huh-7.5 cells were obtained from Pr. Charles M. Rice, Rockefeller University.[40]



Xavier MARTIN-BENLLOCH studied a double diploma in chemistry at the University of Valencia (Spain) and ECPM (Strasbourg, France). He did his doctorate within the Bioorganic and Medicinal Chemistry team at ECPM under the supervision of Dr DAVIOUD-CHARVET and Dr ELHABIRI. After a year as a postdoctoral researcher at the University of Geneva, he decided to enrol in a master course focused on innovation management. He is currently supporting innovative projects in the field of healthcare as a Technology Transfer Officer at Eurasanté.

Thomas PIETSCHMANN is an internationally acknowledged expert in HCV molecular virology and has been working in the field of basic and clinical hepatitis infection research for 20 years. He has co-developed the first fully infectious HCV tissue culture model and used it to characterize the virus host interaction at the molecular level. His focus is on HCV assembly, and cell entry as well as the mechanisms of HCV tissue and species tropism. His team provided major contributions to the understanding of mechanisms of viral evasion from antibodies. He has profiled numerous vaccine candidates in animal models. Thomas PIETSCHMANN is director of the Institute of Experimental Virology at TWINCORE with ca. 40 co-workers. He is also full Professor at the Hannover Medical School. The TWINCORE centre is a joint venture of Hannover Medical School (MHH) and the Helmholtz Centre for Infection Research (HZI). He is member of the Advisory group of the German Society of Virology and was elected to the advisory panel of the president of the Helmholtz Association, the largest non-university research organization in Germany.



Elisabeth DAVIOUD-CHARVET studied Pharmaceutical Sciences, prepared her PhD thesis in Prof. H.-P. Husson's group, at the Institute of Chemistry of Natural Substances, Gif-sur-Yvette, and earned her PhD degree in 1988, in Paris 11 University. In 2001, as visiting scientist, she joined the groups of Prof. G. Kenyon and Prof. C. Williams Jr. in Ann Arbor, at the University of Michigan, and was then invited by Prof. H. Schirmer and Prof. K. Becker as group leader in Biochemie-Zentrum Heidelberg (2002-2011) at Heidelberg University. In 2010, she established her independent group in the research unit UMR7042 CNRS-Unistra-UHA, named „Laboratoire d'Innovation Moléculaire et Applications" (LIMA), located in the European School of Chemistry, Polymers and Materials (ECPM) from Strasbourg University. For more than 25 years she has been focusing on the medicinal chemistry of new redox-active drugs against (neglected) parasitic diseases targeting the redox equilibrium of human parasites.

Mourad ELHABIRI obtained his Ph.D. at Strasbourg University in 1997 in Prof. R. Brouillard's group. During this period, he was interested in the chemistry of anthocyanins and their interactions with metal ions. In 1997, he joined the group of Prof. Jean-Claude Bünzli at EPFL in Lausanne (Switzerland) and worked on the synthesis and photophysical characterization of bimetallic lanthanides triple-stranded helicates. In 1999, he was recruited by the CNRS and focussed his interests on the physicochemical characterization of natural, biomimetic and bioinspired chelators as well as functional supramolecular edifices. During the last 10 years, he has been developing redox-active drugs against viral/parasitic pathogens and elucidating their mechanism of action. He is currently developing innovative functional Vis.-NIR fluorescent dyes for bioimaging.

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Keywords: Flavones • Antivirals • Magnesium(II) Coordination • physico-chemistry • Pharmacokinetic Properties

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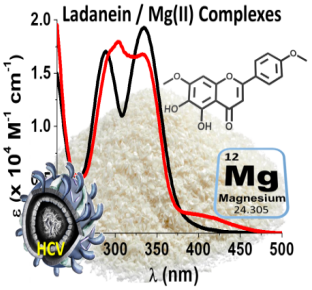

The coordination properties of ladanein, a virucidal flavone, were extensively studied with magnesium(II) precursors prepared from various carboxylic acids. Stable ternary complexes of Mg(II) were characterized and quantified. Mg(II) coordination significantly improved the pharmacokinetic and solubility properties of the antiviral flavone in vitro in the CD-1 mouse model, while maintaining its anti-HCV activity.

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