Nanostructured supramolecular hydrogels: towards the topical treatment of Psoriasis and other skin diseases

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ABSTRACT

Supramolecular hydrogels were synthesized using a bis-imidazolium based amphiphile, and incorporating chemically diverse drugs, such as the cytostatics gemcitabine hydrochloride and methotrexate sodium salt, the immunosuppressive drug tacrolimus, as well as the corticoid drugs betamethasone 17-valerate and triamcinolone acetonide, and their potential as drug delivery agents in the dermal treatment of Psoriasis was evaluated. The rheological behavior of gels was studied, showing in all cases suitable viscoelastic properties for topical drug delivery. Scanning electron microscopy (SEM) shows that the drugs included have a great influence on the gel morphology at the microscopic level, as the incoration of gemcitabine hydrochloride leads to slightly thicker fibers, the incorporation of tacrolimus induces flocculation and spherical precipitates, and the incorporation of methotrexate forms curled fibers. ^{1}H NMR spectroscopy experiments show that these drugs not only remain dissolved at the interstitial space, but up to 72% of either gemcitabine or methotrexate, and up to 38% of tacrolimus, is retained within the gel fibers in gels formed with a 1:1 gelator:drug molar ratio. This unique fiber incorporation not only protects the drug from degradation, but also importantly induces a *Two Phase Exponential* drug release, where the first phase corresponds to the drug dissolved in the interstitial space, while the second phase corresponds to the drug exiting from the gel fibers, and where the speed in each phase is in accordance with the physicochemical properties of the drugs, opening perspectives for controlled delivery.

Skin permeation *ex vivo* tests show how these gels successfully promote the drug permeation and retention inside the skin for reaching their therapeutic target, while *in vivo* experiments demonstrate that they decrease the hyperplasia and reduce the macroscopic tissue damage typically observed in psoriatic skin, significantly more than the drugs in solution. All these characteristics, beside the spontaneous and easy preparation (room temperature and soft stirring), make these gels a good alternative to other routes of administration for Psoriasis treatment, increasing the drug concentration at the target tissue, and minimizing side effects.

INTRODUCTION

Psoriasis has been classified as an immune-mediated inflammatory disease that alters the skin [1], affecting the patients generally with lesions in the form of red scaly plaques scattered over different parts of the body, such as, knees, elbows, back and scalp [2,3]. The chronic nature of the disease is most likely a consequence of a vicious cycle that involves different types of cells and cytokines. For instance, the hyperproliferation of epidermal cells (keratinocytes) secrete autoantigens such as the LL-37 peptide. These autoantigens induce a biochemical cascade involving the activation of immune cells such as dendritic and T cells, and their elevated secretion of cytokines, such as IL-20, IL-23, IL-17, IL-22, and IFN-γ. In turn, these cytokines promote the replication of keratinocytes [3–6]. Many efforts are being made to better understand the complexity of the disease pathogenesis [7], and this gradual understanding has led to the exploration of new therapeutic approaches [8–10]. Over the past few years, immunosuppressant drugs such as tacrolimus, or corticoid drugs such as triamcinolone acetonide and betamethasone 17-valerate, have been dermally applied for treatment of skin diseases, including Psoriasis [11,12]. As well, the cytostatic drug methotrexate has been clinically used as a parenteral or oral treatment for Psoriatic patients not responding to other treatments [3,13]. On the other hand, gemcitabine hydrochloride has been used as a systemic chemotherapeutic agent in different types of cancer, but has not been used as a treatment of Psoriasis. However; it has been reported that, when parenterally administered to cancer patients who were also affected by Psoriasis, a significant decrease of Psoriatic lesions was collaterally observed [14,15]. This observation is most probably a consequence of the drug's ability to prevent the hyperproliferation of keratinocytes, therefore showing its potential in the treatment of this disease. Nevertheless, the long-term applicability of all these drugs, especially those parenterally administered, might be hampered by undesired side reactions. For this reason, the search of better drug delivery systems is of high interest.

Gels can be described as a soft material with viscoelastic behavior, composed of a solid-like network dispersed in a continuous liquid medium [16,17]. This kind of materials can be easily found in our quotidian life, where their properties are mainly determined by their composition and structure at microscopic level [18]. Due to the wide variety of gel types reported nowadays, these are generally classified by the crosslink nature (covalent or non-covalent) [19]. While covalent crosslinking gives rise to rigid structures suitable for devices that require mechanical resistance (e.g. contact lenses) [20], supramolecular aggregations grant these materials with a softer character, applicable in many biomedical fields including drug delivery [21]. Supramolecular gels are generally made of low molecular weight gelators (LMWG), which self-assemble under certain conditions to form the solid-like network. The progress made up to the present day has brought to the literature a wide range of compounds that can act as LMWG [17]. It has been reported that imidazolium compounds can show important host-guest supramolecular interactions, drastically changing their properties such as the pKa.[22] We have proven that dicationic bis-imidazolium amphiphiles can form supramolecular gels, which are able to effectively stabilize and release drugs from their matrix. Moreover, such drugs can be not only anionic [23,24], but also cationic [25], or neutral, as is the case of the lipophilic corticoids betamethasone 17-valerate or triamcinolone acetonide [26], therefore proving their potential as delivery systems for topical application.

In the present work, we tested the ability of the dicationic imidazolium-based amphiphile **1·2Br** (Figure 1) to form gels in presence of drugs which are suitable in the treatment of Psoriasis. Such drugs include the previously studied betamethasone 17-valerate and triamcinolone acetonide, as well as the cytostatic or immunosuppressant drugs gemcitabine hydrochloride, tacrolimus, or methotrexate sodium salt. Gels formed were extensively characterized, from their morphology at the microscopic level to their rheological behavior. Moreover, their ability to release these drugs *in vitro*, as well as their ability to permeate the drugs through human skin *ex-vivo,* was studied. In addition, the amount of drug that can be retained inside the skin after topical application was assessed, as it is where they perform their therapeutic activity. Finally, *in-vivo* experiments in mice showed their efficacy on inhibiting the hyperplasia and tissue degeneration characteristic in Psoriasis.
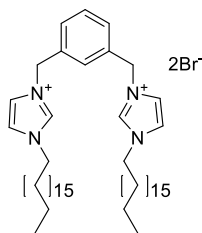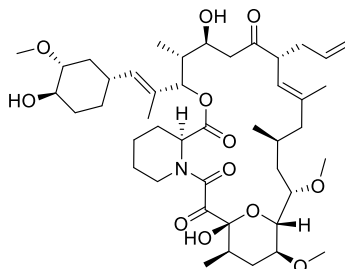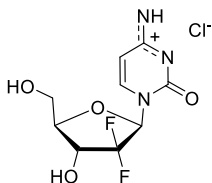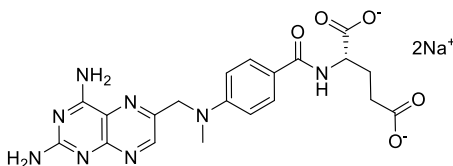
1·2Br

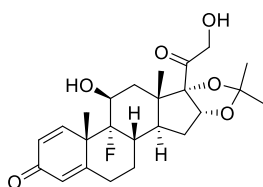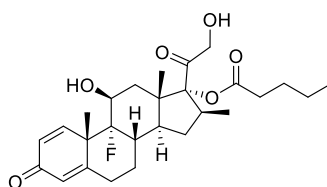**Tacrolimus**

Gemcitabine hydrochloride

Methotrexate sodium salt

Triamcinolone acetonide

Betamethasone 17-valerate

Figure 1. Chemical structures of **1·2Br**, tacrolimus, methotrexate sodium salt, gemcitabine hydrochloride, triamcinolone acetonide and betamethasone 17-valerate.

EXPERIMENTAL SECTION

Materials

All reagents were of analytical grade and were used without further purification processes. Methotrexate, tacrolimus, gemcitabine hydrochloride, sodium dodecyl sulfate and 12-O-Tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma (Germany). Betamethasone 17-valerate and triamcinolone acetonide were purchased from Acofarma (Spain). Paraffin film (Parafilm®) was purchased from Sigma (Germany). Digital caliper was purchased from Truper (Mexico).

Gelling agent 1,3-bis[(3-octadecyl-1-imidazolio)methyl] benzene dibromide (**1·2Br)** was synthetized as reported previously.[27]

Solvents and buffers: water was always used 18mΩ grade from a MilliQ® equipment. Ethanol, acetone (analytical grade), deuterium oxide 99% and methanol-d4 99% were purchased from Sigma (Germany). Phosphate buffer saline (PBS) (tablets) was purchased from Sigma (Germany) and prepared as indicated by the manufacturer.

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Methods

1) Gel Preparation

Gels were always prepared by dissolving compound **1·2Br** in ethanol by means of a sonicator prior to the slowly addition of water as the anti-solvent. Final concentration of **1·2Br** in gels was 5 mg/mL unless stated otherwise. The mixtures were carefully stored in closed vials to prevent solvent evaporation and left to stand without disturbance at room temperature for gel formation.

Gels incorporating a drug were always prepared by first dissolving the drug in either water or ethanol. For gels with tacrolimus (**1·tacrolimus**), betamethasone 17-valerate (**1·betamethasone**), or triamcinolone acetonide (**1·triamcinolone**), the drug was first dissolved in ethanol, the gelator **1·2Br** was added to the solution in solid and sonicated until complete solution was observed. Water was added for completing the gelation process. For gels with gemcitabine hydrochloride (**1·gemcitabine**), drug was dissolved in water and was mixed with an ethanolic solution of gelator **1·2Br** for completing gelation. For gels with methotrexate sodium sat (**1·methotrexate**), a concentrated aqueous stock solution of methotrexate sodium salt was first prepared by adding 1 M NaOH to an aqueous suspension of methotrexate, in a 2:1 molar ratio (NaOH:methotrexate), in order to deprotonate both carboxyl groups from methotrexate molecule and assure its water solubility. Finally, a dilution to the desired drug concentration was made in water, and an ethanolic solution of the gelator **1·2Br** was added to complete the gelation process. A fresh stock solution of methotrexate sodium salt was prepared every time gels were formed.

2) Influence of drug concentration in gel formation

In order to choose the optimal gel composition, 2 mL of each gel with four different final drug concentrations were prepared (1, 2.5, 5 and 10 mg/mL). For the gel with gemcitabine hydrochloride, a solution of **1·2Br** (10 mg) in ethanol (1 mL) was mixed with a solution of gemcitabine hydrochloride (2, 5, 10 or 20 mg) in water (1 mL). For the gel with tacrolimus, **1·2Br** (10 mg) was added to a solution of tacrolimus (2, 5, 10 or 20 mg) in ethanol (1 mL) and was sonicated until dissolved. Finally, 1 mL of distilled water was added in order to complete the gelation process. Gels with methotrexate sodium salt were prepared by mixing an aqueous solution of methotrexate sodium salt (2, 5, 10 and 20 mg of methotrexate) (1 mL), prepared from the methotrexate sodium salt stock solution, with a solution of **1·2Br** (10 mg) in ethanol (1 mL). The optimal gel composition for gels **1·betamethasone** and **1·triamcinolone** was determined according to [26].

Gelation time was measured by means of a tapping-and-tilting method. Briefly, upon mixing ethanolic and aqueous solutions, closed vials were softly tapped at certain intervals, in order to observe the vibration of the

liquid surface. The progressive decrease in the vibration of the surface indicated an increase in viscosity due to gelation. Finally, gelation was confirmed by tilting the vial to observe the sample does not flow.

3) Drug incorporation into gel fibers

Retention of the drug beneath the gel fibers was determined by ^{1}H NMR spectroscopy using a Varian 400 MHz NMR.

For estimating the proportion of either methotrexate sodium salt or gemcitabine hydrochloride incorporated in the gel fibers, two solutions containing equal amounts of drug (2.8 mg, 8.28 μmol) were prepared in two different tubes (A and B), using 0.75 mL of deuterium oxide (D_2O), and a ^{1}H NMR spectrum of each sample was recorded (Record 1). Then, 0.75 mL of deuterated methanol (CD_3OD) with **1·2Br** (7.5 mg, 8.28 μmol) was added to tube A, while in tube B just the 0.75 mL of CD_3OD was added. Agitation of both tubes was necessary in order to ensure a proper homogenization. Gel was formed in tube A, while tube B remained in solution. ^{1}H NMR spectrum of each sample was recorded.

For estimating the proportion of tacrolimus incorporated, two solutions containing equal amounts of drug (6.7 mg, 8.28μmol) were prepared in two different tubes (A and B), using 0.75 mL of deuterated methanol (CD_3OD), and ^{1}H NMR spectrum of each sample was registered. Then, **1·2Br** (7.5 mg, 8.28 μmol) was added to tube A and dissolved. Subsequently 0.75 mL of deuterium oxide (D_2O) were added to both tubes and mixed. Gel was formed in tube A, while tube B remained in solution. ^{1}H NMR spectrum of each sample was recorded.

4) Rheological measurements

Rheological tests of gels **1·2Br, 1·gemcitabine, 1·tacrolimus** and **1·methotrexate** were performed using a a Haake Rheostress1 rheometer connected to a Thermo Haake Phoenix II + Haake C25P temperature control circulating bath, previously set to 32ºC during the whole experiment, and equipped with parallel plate geometry (Haake PP60 Ti, 60 mm of diameter, 2 mm gap between plates). In order to know the viscoelastic behavior of different gels, oscillation rheology testing was performed. Gels were formed in a 3.5 cm-diameter polystyrene plate, having a total volume of 14 mL, at a 5 mg/mL concentration of **1·2Br**. For the drug-loaded gels, a drug concentration of 2.5 mg/mL was used, according to the optimal gel composition chosen. Prepared gels were always kept at room temperature overnight before study. Oscillation amplitude tests were performed by increasing a shear stress from 0.01 to 100 Pa at a constant frequency of 1 Hz, to determine the linear viscoelastic region (LVR). Oscillation frequency tests were performed by increasing the frequency from 0.01 to 10 Hz, at a constant amplitude of 0.5 Pa for being within the LVR. Storage modulus (G') and loss modulus (G") were determined in both tests, which are defined as: $G' = {\tau_0}/{\gamma_0} \cos \delta \quad G'' = {\tau_0}/{\gamma_0} \sin \delta$ (where τ_0 and γ_0 are the amplitudes of stress and strain, respectively, and δ is the phase shift between them). Software Haake RheoWin®Job Manager V.3.3 and RheoWin®Data Manager V.3.3 (Thermo Electron

Corporation, Karlsruhe, Germany) were used to carry out the test and analysis of the obtained data, respectively.

5) Scanning electron microscopy (SEM)

Gels **1·gemcitabine, 1·tacrolimus** and **1·methotrexate** were prepared using a drug concentration of 2.5 mg/mL, according to the optimal gel composition determined, and were mounted on SEM stubs (carbon adhesive), dried using a N_2 current and coated with a sputtered thin film of gold. SEM images were acquired in a Jeol JSM-7100F SEM at 10 kV accelerating voltage at the *Centres Científics i Tecnológics de la Universitat de Barcelona* (CCiTUB). Images were taken at magnifications of 3000x, 10000x and 30000x.

6) Drug release studies

Drug release studies were performed using gels **1·gemcitabine, 1·tacrolimus** and **1·methotrexate**, in a Microette diffusion test system (Microette plus-Hanson Research) following previously reported methodologies [28,29]. Briefly, vertically assembled Franz-type diffusion cells (Crown Glass) (2.54 cm^2 diffusion area) as well as dialysis membranes (CelluSep T3 dialysis membrane, MWCO 12,000–14,000 Da, MFPI, USA), were used. Cells were equipped with a circulating bath previously set to 32 ºC for being the temperature of the skin. For **1·gemcitabine** and **1·methotrexate** release experiments, clean and dry cells were filled with 10 mM PBS pH 7.4 in the receptor chamber, whereas a mixture of ethanol:water (7:3) was used for tacrolimus, always complying SINK conditions [30]. The dialysis membranes were previously hydrated with the receptor solution and were mounted between the matched donor and the receptor compartment. Finally, a known amount of gel (≈0.3 g) was placed on the membrane surface in the donor compartment. All Franz cell openings including donor top and receptor arm were occluded with paraffin film to prevent evaporation. The receptor compartment was stirred at a constant speed using a magnetic stirrer. Sample volumes (300 µL) were taken at certain intervals and stored at -20 ºC for further analysis. Samples taken were immediately replaced by fresh receptor medium in order to maintain a constant volume and assure SINK conditions. All experiments were performed by triplicate for over 75 h. Drug concentration in samples was determined by HPLC.

7) Skin permeation studies

Human excised skin was obtained during abdominal plastic surgery on a 40-year-old Caucasian female with informed consent and appropriate institutional ethical approval. Full sections of 0.4 mm thickness containing the epidermis and part of the dermis were separated by means of an electric dermatome and stored at −20°C until required. *Ex vivo* human skin permeation experiments were conducted following a protocol similar to that in drug release section, but replacing the dialysis membranes by the skin sections with the *stratum corneum* facing the donor compartment according to guidelines [31,32]. Gels **1·gemcitabine, 1·tacrolimus, 1·methotrexate, 1·betamethasone** and **1·triamcinolone** were introduced into the donor chamber in contact

with the epidermal side of the skin. The experiment was conducted over 27 h in order to prevent damage of the biological material. Samples (300 μL) from the receptor chamber were withdrawn at appropriate time intervals and stored at -20 ℃ for further HPLC analysis, and the withdrawn volume was immediately replaced by receptor solution. All skin permeation studies were done by triplicate.

8) Drug extraction from the skin

At the end of the permeation study, the skin was cleaned with a gauze soaked in a 0.05% solution of sodium dodecyl sulfate and rinsed with water accurately. Then the skin outside of the permeation area was cut off and the permeation area was perforated with a needle, weighed, immersed in 1 mL of its corresponding receptor medium, and sonicated during 20 minutes in order to extract the drug from the skin. The resulting solutions were stored at -20 ℃ for further HPLC analysis.

9) Drug recovery experiments

In order to know the maximum amount of drug that can be extracted from the skin, 2.5 mg/mL drug solutions (methotrexate sodium salt, or gemcitabine hydrochloride or tacrolimus or betamethasone 17-valerate or triamcinolone acetonide) were prepared. An aliquot of each drug solution was stored at -20 ℃ (*original* solution). Slices of skin were incubated separately inside Eppendorf tubes filled with a second aliquot of each drug solution for 24 hours at 32 ℃. Afterwards, the skin was taken out and the solution was stored at -20 ℃ (*after-incubation* solution). Drug was extracted from the skin following the same protocol (Drug extraction from the skin) and the resulting solution was stored at -20 ℃ (*extraction* solution). Drug concentrations of all samples were determined by HPLC. The drug retained inside the skin (absorbed by the skin) was obtained from the difference between *original* and *after-incubation* solutions. The recovery factor was calculated as the drug extracted divided by the drug retained inside the skin.

10) HPLC determination

The HPLC system used consisted of a Waters 2695 pump + injector, with a 2996 Photo Diode Array, equipped with an Ultrabase® 100 Å, 5 μm pore, ODS2 column (150 mm X 4.6 mm) from AKADY® Cromatográfica (Barcelona). Analytical methods used were previously validated.

For the analysis of tacrolimus, the aqueous phase consisted of 40 mM sodium acetate with 85 μL/L of acetic acid in MilliQ water (pH 6). The aqueous solution was filtered before use (Channel A). The organic phase consisted of methanol (HPLC grade) (Channel B). The mobile phase consisted of a mixture of Channel A:Channel B, at a flow rate of 1 mL/min, following a gradient starting at 30:70 at 0 min, to 0:100 at 10 min, kept until 15 min, to 30:70 at 16 min, and kept until 22 min for stabilizing the column. Samples were monitored and further analyzed at 205 nm wavelength.

For the analysis of gemcitabine, the mobile phase consisted of a mixture of water-methanol, at a flow rate of 1 mL/min, following a gradient starting at 95:5 at 0 min, to 10:90 at 4.5, to 95:5 at 5.5 min, and kept until 10 min for stabilizing the column. Samples were monitored and further analyzed at 268 nm wavelength.

For the analysis of methotrexate, the aqueous phase consisted of 40 mM sodium acetate with 85 µL/L of acetic acid in MilliQ water (pH 6). The aqueous solution was filtered before use (Channel A). The organic phase consisted of methanol (HPLC grade) (Channel B). The mobile phase consisted of a mixture of Channel A-Channel B, at a flow rate of 1 mL/min, following a gradient starting at 95:5 at 0 min, to 60:40 at 4.5 min, kept until 7 min, to 30:70 at 8 min, kept until 10 min, to 95:5 at 11 min, and kept until 15 min for stabilizing the column. Samples were monitored and further analyzed at 300 nm wavelength.

Calibration curves of all drugs were analyzed, and concentrations of all samples were determined constructed on the basis of peak area measurements.

11) Data processing

For drug release, skin permeation, drug extraction from the skin, and recovery experiments, data obtained by HPLC were treated using Microsoft Excel®. For drug release experiments, cumulative percentage of drug released was plotted against time, and different non-linear regression models including *Higuchi*, *Weibull*, *Korsmeyer-Peppas*, *One Phase-* and *Two Phase Exponential Association models* were tested. The best fitting model was chosen by means of the R^2 value, through which kinetic parameters were obtained for each type of gel. For skin permeation studies, cumulative amounts of drug permeated to the receptor chamber were plotted versus time, and linear regression analyses were performed for obtaining the kinetic parameters. All regressions (linear and non-linear) were performed using GraphPad Prism (version 3.00, GraphPad software, Inc., USA).

12) In-vivo experiments

Male Swiss CD-1 mice (20–25g) were purchased from Circulo ADN S.A. de C.V. (Mexico) and were subjected to a quarantine period of 7 days on arrival. The animals were housed in plastic cages with soft bedding with access to controlled diet and optional tap water. The temperature was kept at 24±1∘ C and the relative humidity was kept at 50–60%. Artificial lighting was used to provide 12h light and 12h dark every 24h. The studies were conducted under a protocol in accordance with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999).

Inflammation and hyperplasia was induced in groups of mice (n=6) by daily applying a solution of 12-O-Tetradecanoylphorbol 13-acetate (TPA) (2.5 µg) in ethanol (5 µL) along ten days on both sides of the right ear, while applying only ethanol on the left ear (without TPA) as a Control(-) (healthy skin). At the same time, on days 2, 4, 6, 8 and 10, a treatment was applied to each group of mice on both sides of the right ear. Treatment applied was either gel **1·2Br** or **1·betamethasone** or **1·triamcinolone** or **1·tacrolimus** or

1   **1·methotrexate,** or a drug in solution betamethasone 17-valerate or triamcinolone acetonide or tacrolimus

2   in acetone, or methotrexate sodium salt in water, at the same concentrations than in gels. A group of mice

3   was treated with the cream Celecrem® containing bethamethasone 17-valerate as the reference marketed

4   product. A group of mice with daily TPA application on the right ear but without further treatment was taken

5   as Control(+) (psoriatic skin). In addition, to evaluate the effect of the gel alone on healthy skin, a group of

6   mice was treated with gel **1·2Br** on the right ear without TPA application. At days 5, 8 and 11, pictures of

7   the mice ears were taken, the main signs at the macroscopic level were observed, and the thickness of both

8   ears was measured using a digital caliper. The thickness increase relative to Control(+) (psoriatic skin) was

9   calculated according to the equation:

10

11   *Relative increase* (%)

12
$$= \frac{Thickness\ right\ ear\ [treatment] - Thickness\ left\ ear\ [treatment]}{Thickness\ right\ ear\ [Control(+)] - Thickness\ left\ ear\ [Control(+)]}\ 100\%$$

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RESULTS AND DISCUSSION

Optimization of gel formation

The gelation behavior of supramolecular gelators is determined by different variables such as temperature, nature of solvent, and type and concentration of a drug in the gel [23,33]. As the cationic compound **1·2Br** can form supramolecular gels in water-ethanol mixtures [26], here we evaluate the influence that the drugs gemcitabine hydrochloride, tacrolimus, and methotrexate sodium salt have on the gelling process, by observing the gelling time at different drug concentrations. The concentration of the gelator **1·2Br** was maintained to 5 mg/mL for being the optimal for preparing gels for dermal drug delivery [23,26]. As it can be seen in Figure 2, the gel **1·2Br** (without drug) is spontaneously formed in 10 min at room temperature, in accordance with previous observations [26]. As well, in the gels containing a drug, the increase of drug concentration results in an increase of the gelling time, independently of the type of drug. In the case of gel **1·gemcitabine**, the lowest drug concentration does not change the gelling time (10 min) as compared to gel without drug; however, the incorporation of either tacrolimus or methotrexate sodium salt at low concentrations seems to decrease the gelling time, forming gels in less than 10 min. For instance, at 2.5 mg/mL drug concentration, gel **1·methotrexate** is formed in 7 min, and gel **1·tacrolimus** is formed in only 3 min. Also, at the rest of the concentrations tested, gel **1·tacrolimus** showed the fastest gelling. These results suggest that, when dissolved in the ethanol:water medium at low concentrations, either methotrexate or tacrolimus can act as a template, enhancing the nucleation to fiber formation. However, as the concentration of the drug is gradually increased, the steric hindrance of the drug molecules may impede the interaction between gelator-gelator molecules leading to the fibers growth, which explains the increase in gelation time. It is remarkable that this templating behavior is also observed with triamcinolone acetonide; however, it is not observed with other drugs such as gemcitabine, betamethasone, brimonidine, or AEBSF HCl, at the lowest drug concentrations tested [25,26].
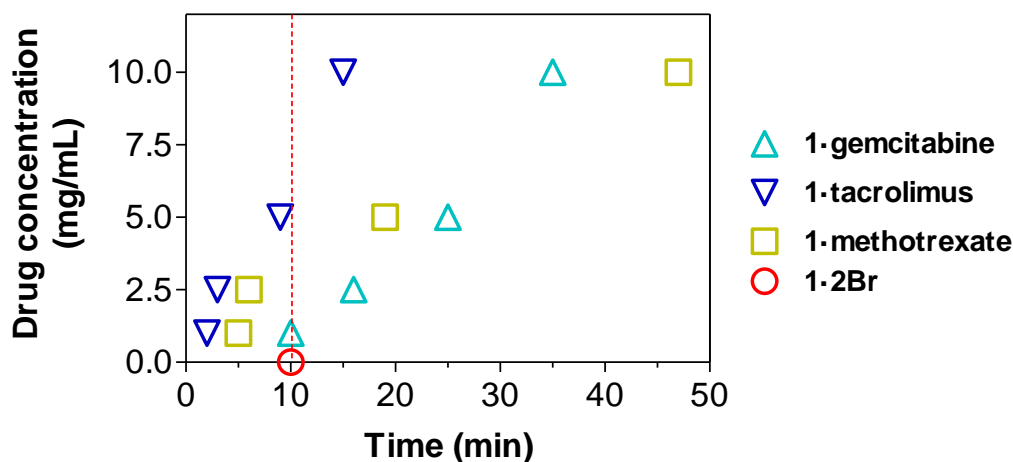
Figure 2. Influence of drug concentration on gelling times for gels **1·2Br** (O), **1·gemcitabine** (Δ), **1·tacrolimus** (∇) and **1·methotrexate** (□).

Optimum conditions for gel formation were therefore chosen considering a macroscopic homogeneous appearance, a sufficient drug concentration for therapeutic purposes, and a relatively fast gelling time (<20 min). For instance, at the macroscopic level, no appreciable differences were observed between the different samples tested. Regarding gelling time, gels with a 2.5 mg/mL drug concentration or lower, formed gels in 16 min, 3 min and 6 min, for **1·gemcitabine**, **1·tacrolimus** and **1·methotrexate**, respectively. Regarding previously reported or commercially available formulations, it can be seen that Protropic® (tacrolimus ointment 0.3 mg/g or 1 mg/g), Positon® (ointment containing triamcinolone acetonide 2.5 mg/g), or other non-commercial products reported in literature (methotrexate ointments 1 mg/g to 30 mg/g) [34–36], show in all cases drug concentrations similar or lower than 2.5 mg/mL. Therefore, 2.5 mg/mL was chosen as the optimum drug concentration, at which gels were prepared in the rest of the experiments, unless stated otherwise.

For gels including betamethasone 17-valerate (**1·betamethasone**) or triamcinolone acetonide (**1·triamcinolone**), optimum conditions had been determined as a 5 mg/mL drug concentration and a 5 mg/mL gelator concentration. At these conditions, gel **1·betamethasone** is formed in 10 min at room temperature, while **1·triamcinolone** is readily formed in only 2 minutes [26].

Rheological analysis

In order to evaluate the influence of the drug in the viscoelastic behavior of the gels, oscillatory analyses were carried out in gels **1·2Br**, **1·gemcitabine**, **1·tacrolimus** and **1·methotrexate.** Amplitude sweep tests show that the phase angle for all samples across the Linear Viscoelastic Region (LVR) was relatively low (17º - 20º). As well, G' values are higher than G'' ones in all samples. Results in these two parameters indicate

that the elastic (solid-like) component is prevalent over the viscous (fluid-like) one (Figure 3). Frequency sweep tests were performed in these gels at a constant shear stress of $\tau = 0.5$ Pa for being within the LVR (See Supporting Information, Figure S 1), finding also that, independently of the frequency applied, G' is higher than G'', confirming the solid-like behavior of these gels [37,38].
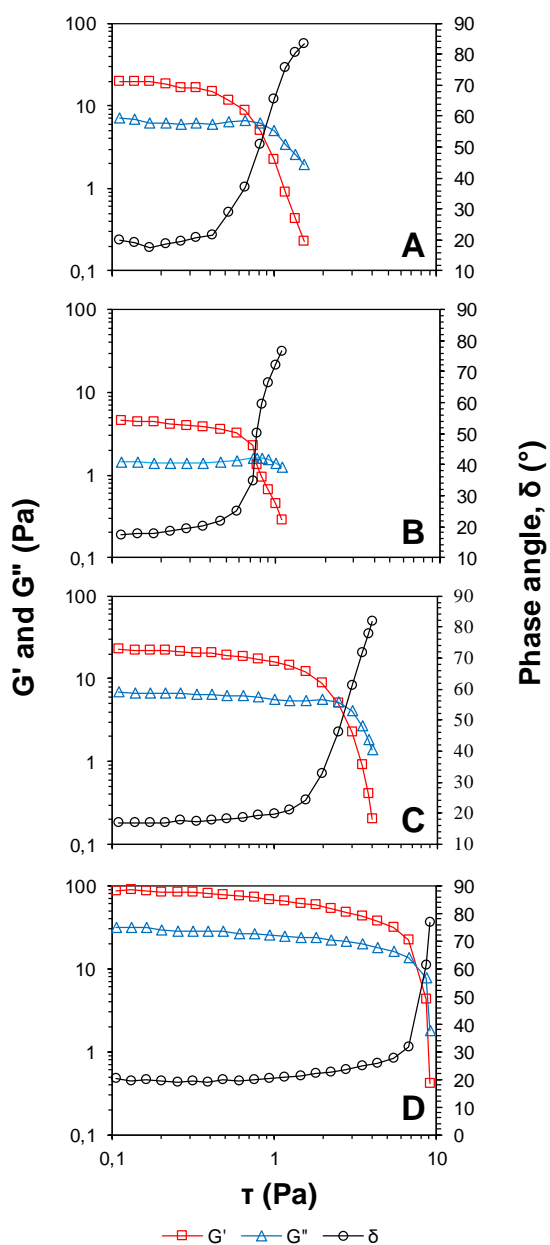
Figure 3. Elastic modulus (G'), loss modulus (G'') and phase angle (δ) as a function of shear stress: (A) **1·2Br** (B) **1·gemcitabine**, (C) **1·tacrolimus**, and (D) **1·methotrexate**.

Table 1. Critical stress values from gels with and without drug.

Gel	Critical stress (Pa)
1·2Br	0.415
1·gemcitabine	0.576
1·tacrolimus	1.237
1·methotrexate	5.385

- 13 -

Amplitude tests also show the critical stress, which can be identified as a sharp drop in G', and which is the minimum stress that overcomes the elasticity or inherent resistance to rupture within the sample. The resulting critical stress values (f = 1Hz) for each sample are shown in Table 1, where it can be seen that the elasticity of the material is increased when incorporating either of these drugs. For instance, gel **1·gemcitabine** is 1.38 times more resistant to rupture, gel **1·tacrolimus** is almost 3 times more resistant, and gel **1·methotrexate** is almost 13 times more resistant, than gel **1·2Br**. Conversely, the incorporation of the neutral corticoid drugs betamethasone 17-valerate or triamcinolone acetonide, or other cationic or anionic drugs into gel **1·2Br**, has resulted in a loss of elasticity.[26] Nevertheless, it is worth noticing that the incorporation of either drug (gemcitabine hydrochloride or tacrolimus or methotrexate sodium salt, or even triamcinolone acetonide or betamethasone 17-valerate[26]) always leads to gels that can be broken abruptly when critical stress is reached, which is optimum for a comfortable application on irritated skins. On the other hand, the softness of the material, as seen in the resistance to deformation (G' and G'' values) is also strongly influenced by the drug added. However, no direct relationship can be apparently observed between this property and drug molecular weight, charge, or amount of drug incorporated in fibers. For instance, the inclusion of either triamcinolone acetonide or tacrolimus does not change the resistance to deformation as compared to gel **1·2Br**, while the addition of either betamethasone 17-valerate or gemcitabine hydrochloride makes it four-times softer, and while the addition of methotrexate sodium salt makes it eight-times more rigid.

Drug incorporation into gel fibers

^{1}H NMR experiments were performed to know if gels formed with compound **1·2Br** incorporate either gemcitabine hydrochloride or tacrolimus or methotrexate sodium salt dissolved in the interstitial space of the gel, or if they can even incorporate these drugs within gel fibers. For instance, when a ^{1}H NMR spectrum is collected from a gel, signal only arises from species dissolved in the liquid trapped between the fibers, and intensity values of intra-molecular resonances depend upon the number of nuclei causing the signal [39]. Thus, as the total concentration of drug used to form the gel is known, it is possible to assess the proportion of drug that is trapped inside the gel fibers when the gel is formed. Indeed, results show that these three drugs are not only retained in the interstitial space of the gel but also beneath gel fibers. Experiments were performed at a 1:1 gelator:drug molar ratio for quantification purposes, finding that gel **1·2Br** can incorporate up to 72% of either methotrexate or gemcitabine, and up to 38% of tacrolimus inside gel fibers (See Supporting Information, Figure S 2 to Figure S 4). As well, gel **1·2Br** in presence of triamcinolone acetonide or betamethasone 17-valerate in a 1:1 molar ratio, entraps up to 54% and 65% of the drug inside the fibers, respectively [26].

Therefore, according to the optimum drug concentration (see Optimization of gel formation), when gels **1·gemcitabine**, **1·tacrolimus**, and **1·methotrexate** are prepared with a 2.5 mg/mL drug concentration, 48% (1.19 mg/mL) of gemcitabine, 68% (1.69 mg/mL) of tacrolimus, and 72% (1.81 mg/mL) of methotrexate, should be incorporated inside the fibers. Similarly, when gels **1·triamcinolone** and **1·betamethasone** are formed with a 5 mg/mL drug concentration, 26% (1.29 mg/mL) of triamcinolone, and 34% (1.71 mg/mL) of betamethasone, should be entrapped in fibers [26].

Only a few examples of drugs incorporated inside fibers can be found in literature, but in all cases, either the fabrication process or the drug release shows drawbacks. For instance, fiber-incorporation has been achieved through electrospinning techniques in polymeric gels; nonetheless, the control of several parameters is required, both of the equipment (electric field, air velocity in spinning chamber, flow rate), and of the component that forms the fibers (dielectric constant, surface tension, and conductivity) [40–43]. On the other hand, the entrapment of a drug in the fibers of a supramolecular gel has been reported; however, it requires the previous covalent attachment of the drug to the gelator, and therefore its release depends on an enzymatic cleavage [44]. Instead, in almost the totality of the gels containing drugs, the drugs are dissolved in the interstitial space. For this reason, supramolecular gels formed with compound **1·2Br** constitute a multiple advantage as a drug delivery system: a) the non-covalent bonds forming the fibers of the supramolecular gels makes them soft and therefore suitable for dermal application; b) the entrapment of the drugs in the fibers provides a reservoir, storing and protecting the drug from degradation; c) such entrapment is non-covalent, making the release independent of the presence of enzymes; and d) the preparation of these drug-loaded gels is easy and fast, as it occurs spontaneously at room temperature in a few minutes. All these characteristics make these gels a promising material for controlled delivery.

Scanning Electron Microscopy

The incorporation of a drug can strongly influence the nanometric structure of gel **1·2Br** fibers. For this reason, the role that played the incorporation of either gemcitabine hydrochloride, tacrolimus or methotrexate sodium salt on the xerogel structure was revealed by Scanning Electron Microscopy (SEM) and compared to that of **1·2Br** alone.

The xerogel of **1·2Br** presents a packed structure with long cylindrical fibers around 140 nm in diameter (Figure 4, A, B), in agreement with previous observations [26].
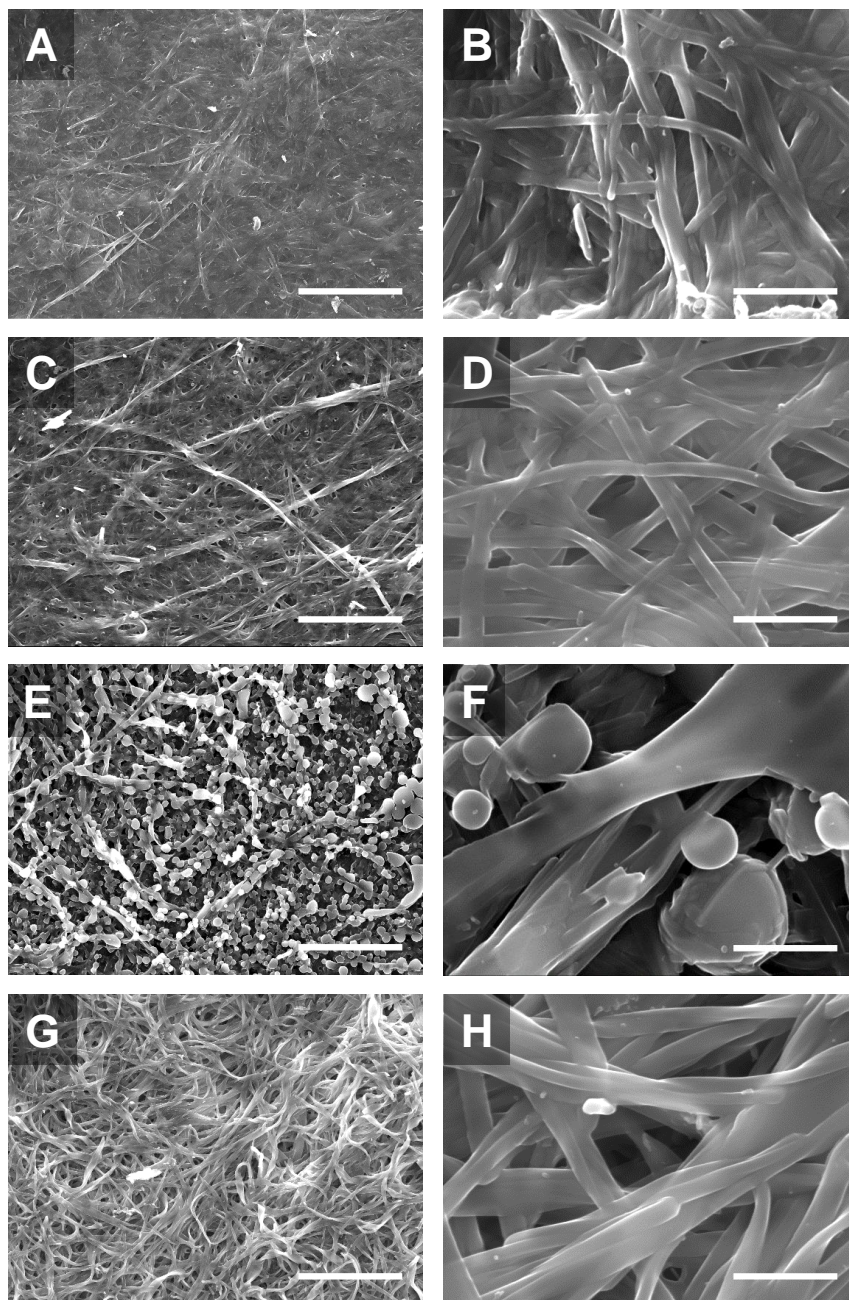
Figure 4. SEM images of xerogels of (A,B) **1·2Br**, (C,D) **1·gemcitabine**, (E,F) **1·tacrolimus,** and (G,H) **1·methotrexate**. Scale bar represents 10 µm and 1 µm for the left and right images respectively.

1·gemcitabine xerogel network is also formed by long fibers, though they seem to be slightly bigger in diameter (200 nm), and the spaces between the fibers seem to be slightly wider (Figure 4, C, D). It is interesting to mention that, as shown by ^{1}H-NMR drug incorporation experiments, when gels are prepared with a 2.5 mg/mL, 52% of the drug remains dissolved at the interstitials; therefore, upon solvent evaporation in sample preparation, observations of drug precipitates in between the fibers would be expected. However, xerogel of **1·gemcitabine** shows no drug precipitates, as compared to a solution of gemcitabine hydrochloride under the same conditions but without gelator, where needle-shaped crystals are found after drying the solvent (See Supporting Information, Figure S 5, A). This suggests that the gelator **1·2Br** might be promoting gemcitabine hydrochloride to precipitate in nanometric structures that cannot be seen under the equipment

resolution. This behavior had been previously observed when incorporating another cationic drug (AEBSF·HCl) into gel **1·2Br.**[25] Such important decrease in particle size is highly desirable for therapeutical purposes, as it increases the total drug surface area and thus the speed of dissolution for reaching the pharmacological target.

On the other hand, xerogel **1·tacrolimus** shows a heterogeneous structure composed by spherical drug precipitates adhered over the gel fibers (Figure 4, E, F). Acordingly, tacrolimus in ethanol:water (50:50) solution forms spherical amorphous precipitates when dried (See Supporting Information, Figure S 5, B), for which spherical precipitates in the xerogel most probably correspond to drug. This morphology had also been observed with gels incorporating betamethasone 17-valerate, which is also a neutral drug. Conversely, xerogels with triamcinolone acetonide, despite also being a neutral corticoid drug, show prismatic-shaped drug precipitates [26]. This suggests that the precipitation in amorphous spherical forms is probably promoted by higher lipophilicities, as both tacrolimus and betamethasone 17-valerate show logP values around 3.5, while triamcinolone acetonide shows a logP value of 2.5 (See Supporting Information, Table S 2). Also, the xerogel fibers of **1·tacrolimus** show a wider dispersity in size as compared to **1·2Br**, with diameters ranging from 170 nm to 1 μm, showing flocculation, where fibers stick together to form thicker fibers.

Very differently, xerogel **1·methotrexate** presents a characteristic morphology of curled single fibers of around 120 nm thick. Flocculation can also be observed, as the individual fibers attach longitudinally, forming thicker fibers varying from 250 to 500 nm in width. As well, bigger interstitial spaces are created by the fiber curls (Figure 4, G, H). Interestingly, an ethanol:water (50:50) solution of methotrexate sodium salt but without the gelator, after drying the solvent, shows precipitates also in the shape of curled fibers. A possible explanation of this behavior is that the majority of long chiral molecules bearing a stereocenter tend to form twisted aggregates [45–47]. Drug incorporation experiments show that, at a 2.5 mg/mL drug concentration, up to 72% of the drug in the gel is incorporated inside the fibers. Thus, as xerogels of **1·methotrexate** show a continuous curled morphology, some of these curls could possibly be comprised of drug exclusively, but some of them should be comprised of drug-loaded gel fibers. This shows that the methotrexate sodium salt incorporated inside the fibers is promoting an important change of the shape of the fibers, from a somehow straight shape, into a clearly curled one that provides wide interstitial areas.

All these results not only confirm that changes among the guest substance lead to changes in gel morphology, as reported by other authors [48], but most importantly, these observations provide consistent explanations for the rheological behavior of gels. For instance, the entangled curled structure of **1·methotrexate** could explain both the higher elasticity (higher critical stress values) and the higher resistance to deformation (higher G' and G'' values) as compared to **1·2Br**. Similarly, the wider fibers created in **1·tacrolimus** might be responsible for the increase in elasticity.

Drug release studies

In vitro release experiments of gels **1·gemcitabine, 1·tacrolimus** and **1·methotrexate** were performed to demonstrate that the gel **1·2Br** matrix is able to release the drug incorporated in it, and that the release profile does not limit the permeation of the drug through the human skin. Cumulative percent of either gemcitabine or tacrolimus or methotrexate released from gel matrix over 3 days is shown in **Error! Reference source not found.**. As well, experimental data from release experiments of **1·betamethasone** and **1·triamcinolone** were taken from [26] and plotted in the figure for comparison purposes. Results from the release of gel **1·tacrolimus** show bigger variabilities as compared to the other gels, which is due to the lack of a chromophore group in tacrolimus molecule, making its detection under UV-absorbance HPLC more difficult.
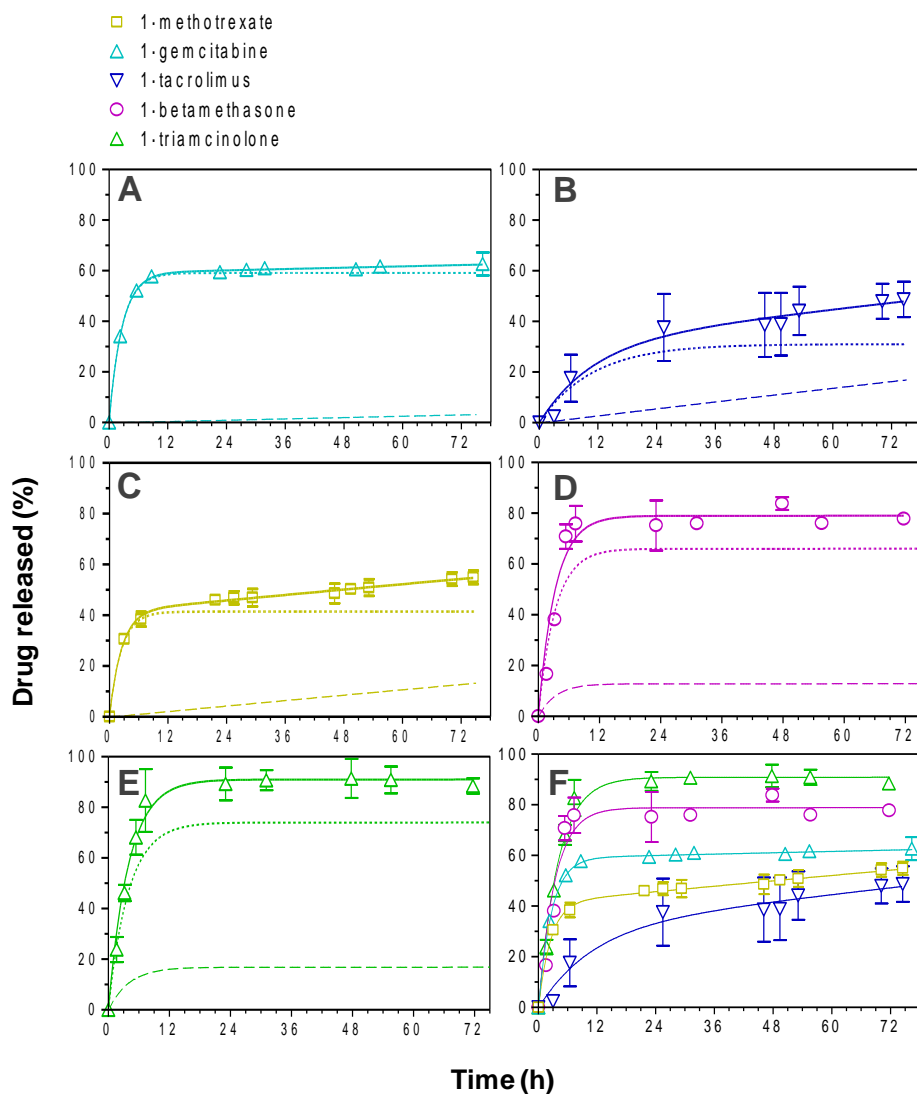
Figure 5. *In vitro* drug released (%)along time from gels (A) **1·gemcitabine,** (B) **1·tacrolimus,** (C) **1·methotrexate,** (D) **1·betamethasone** and (E) **1·triamcinolone.** Experimental data from **1·betamethasone** and **1·triamcinolone** were taken from [26]. Drug release in all cases followed a *Two-Phase Exponential Association* model. The first phase release is shown in dotted line, the second phase release is shown in dashed line, and the cumulative amount of drug released from both phases is shown in continuous line. (F) A comparative view is shown. Values represent Means ±SD (n=3).

Different models were fit within the release data using non-linear-least squared regression, while the best model was chosen by means of the R^2 value. Gels **1·gemcitabine, 1·tacrolimus** and **1·methotrexate** best followed a *Two-Phase Exponential Association* model, which is described by the equation:

$$Y = Y_{max1}(1 - e^{-K1\,t}) + Y_{max2}(1 - e^{-K2\,t})$$

Where the total amount of drug released (*Y*) at a certain time (t), is the result of two exponential drug release processes occurring at the same time, each one at a certain speed (*K*), and with a maximum amount of drug released (*Y_max*). It has been reported that the morphology of the gel at the microscopic level is a main factor influencing the release profile; for example, an open-porous matrix morphology facilitates the drug diffusion, causing an initial burst [49–52]. However, in these supramolecular gels, the matrix morphology is not the only factor influencing the *Two-Phase* release behavior. Instead, the proportion of drug that is outside and inside the gel fibers is most probably the factor playing a major role. In this sense, the *first phase*, or faster drug release, might correspond to the drug that is dissolved in the interstitial space, and is released from the matrix by diffusion through the pores; while the *second phase*, or slower release, could correspond to the drug initially entrapped inside the fibers, which first exits to the interstitial space, and then diffuses through the pores.

As shown in the drug incorporation experiments, in gels with a 2.5 mg/mL drug concentration, approximately 48% of gemcitabine, 72% of methotrexate, and 68% of tacrolimus should be inside the fibers, for which 52%, 28% and 32%, respectively, should be dissolved in the interstitial space of the gel. Accordingly, drug release experiments show that nearly 59% of gemcitabine, 41% of methotrexate, and 31% of tacrolimus can be released in the *first phase*, as can be seen by the Y_{max1} values, and therefore in consonance with the drug dissolved in the interstitial space. Drug release parameters such as maximum drug released and speed constants are shown in Supporting Information (Table S 1). As the *first phase* release occurs by diffusion through the solvent, the speed may be influenced by both the affinity of the drug for the medium, and the porosity of the gel matrix. Results show that both gemcitabine and methotrexate are released four-times faster than tacrolimus, as can be seen in the speed constant (K1) values, suggesting that the highly hydrophobic nature of tacrolimus, and its low water solubility, could influence a slower diffusion through the medium at interstitial space as compared to methotrexate and gemcitabine. Partition coefficient (LogP), water solubility values, and other drug physicochemical factors are shown in Supporting Information (Table S 2).

In the *second phase*, in contrast, the three gels keep releasing drug at a significantly lower speed as compared to the *first phase*. It can be seen that nearly 7.3 µg of tacrolimus per hour per mL of gel can be released, while methotrexate is released at 3.8 µg h^{-1} mL^{-1}, and gemcitabine at 1.5 µg h^{-1} mL^{-1}. As the *second phase* occurs by diffusion through the gel fibers until reaching the interstitial space, the speed of release may be influenced by the concentration gradient between the fibers and the interstitials, as well as by the drug's

affinity for both the fibers and the interstitial space. Hence, as the gelator molecule bears hydrophobic carbon chains, hydrophobic drugs may diffuse through the fibers to reach the interstitials, while highly hydrophilic drugs, once entrapped within the fibers, may be retained inside for longer periods by hydrophobic interactions. Accordingly, the hydrophobic structure of tacrolimus may be related with its faster release in the *second phase*, as compared to that of methotrexate and gemcitabine, which show a highly hydrophilic nature.

This *Two-phase* release mechanism can also explain the release profile observed in gels **1·triamcinolone** and **1·betamethasone**. Although the release of these two gels has been described as a *One-Phase Exponential Association* model [26], it can indeed be described as a T*wo-Phase* model, where the speed constants of the first (K1) and second (K2) phases are similar. According to the drug incorporation experiments, at a 5 mg/mL drug concentration, approximately 74% of triamcinolone acetonide and 66% of betamethasone 17-valerate should be dissolved in the interstitials. Besides, drug release experiments show that 91% of triamcinolone acetonide and 79% of betamethasone 17-valerate is released during a period of 12 hours [26]. Correspondingly, the release profiles of both drugs accurately comply a *Two-phase exponential association* model with R^2 values of 0.99 and 0.98, respectively (See Supporting Information, Table S 1). This suggests that the 91% of triamcinolone released during the 12-hour period most probably corresponds to a first phase, where the drug dissolved at the interstitials (74%) is released, plus a second phase, where 17% of the drug exits from the fibers, both phases showing speed constants (K1=K2) of 0.25 h^{-1}. Similarly, the 79% of betamethasone released during the first 12 hours might correspond to a first phase, where the drug dissolved in the interstitials (66%) is released, plus a second phase, where 13% of drug exits from the fibers, with speed constants of 0.3 h^{-1}. These results are in accordance with the physicochemical properties of betamethasone 17-valerate and triamcinolone acetonide, which may influence their faster release from the gel fibers as compared to the other drugs. For instance, the log P values of these two drugs show their moderate lipophilic nature, which explains their diffusion through the fibers. Subsequently, their solubility in water, up to three orders of magnitude higher than that of tacrolimus, could explain a higher affinity for the hydroalcoholic medium at the interstitials, promoting their exit from the fibers and further diffusion through the medium. This in turn creates a high concentration gradient between the fibers and the interstitials, promoting more drug release from the fibers. In contrast, the hydrophobicity of tacrolimus might explain its diffusion through the fibers; however, its low water solubility might retard its exit to the interstitials for further diffusion, creating a lower concentration gradient between the fibers and the interstitials, and promoting a slower release.

All these results show that the unique capability of these supramolecular gels for incorporating drug beneath its own fibers, acts not only as a protection against drug degradation, but also makes this material a promising platform for controlled release, behaving selectively according to the nature of the drugs.

Skin permeation assays

For the topical treatment of Psoriasis, two steps are crucial in the biochemical pathway of the disease, the autoimmune response, and the uncontrolled replication of keratinocytes in the epidermis *stratum basale* [4–6]. Thus, when either methotrexate or gemcitabine are used for the treatment of dermatological diseases such as Psoriasis, their pharmacological target is located at the epidermis, as both drugs are intended to inhibit replication of cells of the *stratum basale*. In case of methotrexate, this occurs by inhibiting dihydrofolate reductase enzyme, while in case of gemcitabine, by inhibiting ribonucleotide reductase enzyme. In contrast, the pharmacological target of tacrolimus is located both at the dermis and the epidermis, where it inhibits the activity of immune cells such as T lymphocytes [14,53,54]. For this reason, in order to achieve their pharmacological efficacy, all of these drugs should penetrate the *stratum corneum* when topically applied, and ideally be retained beneath the epidermal and dermal layers. This would imply an increase in the concentration at the target tissue, and subsequently a raise in efficacy. Moreover, this retention would act as a drug depot, providing therapeutic activity for longer periods.

For this reason, skin permeation experiments with gels **1·gemcitabine, 1·tacrolimus** and **1·methotrexate** at a drug concentration of 2.5 mg/mL were performed by placing them over human skin, using the same vertical diffusion cell system used in drug release experiments, but replacing the dialysis membrane for human skin, in order to quantitatively evaluate skin permeation parameters. Skin permeation experiments with gels **1·triamcinolone** and **1·betamethasone** had been performed before by us, showing that both drugs triamcinolone acetonide and betamethasone 17-valerate, when incorporated in gel **1·2Br**, can penetrate the skin and remain retained beneath the epidermal and dermal layers, to a higher extent than existing products on the market Celecrem®, containing betamethasone 17-valerate, and Positon®, containing triamcinolone acetonide [26]. Nonetheless, due to the great biological variability between patients, permeation experiments with gels **1·betamethasone** and **1·triamcinolone** were also conducted in this work using human skin from the same patient for comparison purposes, using a drug concentration of 5 mg/mL for being the optimum concentration in these cases. At the end of the study, a skin extraction was performed to evaluate the amount of drug that was retained inside the tissue.

Cumulative amounts of permeated drug per surface area [A] (μg/cm^2) were plotted against time [t] (h) (Figure 6, A) and skin permeation parameters can be seen in detail in Supporting Information (Table S 3). From the drugs studied, neither gemcitabine nor tacrolimus were detected in the receptor medium, showing they were not able to permeate the skin completely, while the rest of the drugs did show complete permeation. The inability of gemcitabine and tacrolimus for permeating the skin is most probably a consequence of their physicochemical properties. Parameters such as partition coefficient (log P) (measure of the lipophilic nature) and molecular weight (MW) are commonly used for predicting the skin permeability of a drug, being those

1   compounds with a log P ranging from 1 to 3 and a MW under 500 g/mol the ones which should present good

2   skin permeation.[55,56] Gemcitabine is therefore significantly more hydrophilic than the ideal range,

3   whereas tacrolimus has a MW of 804 g/mol, much higher than the ideal size (See Supporting Information,

4   Table S 2).
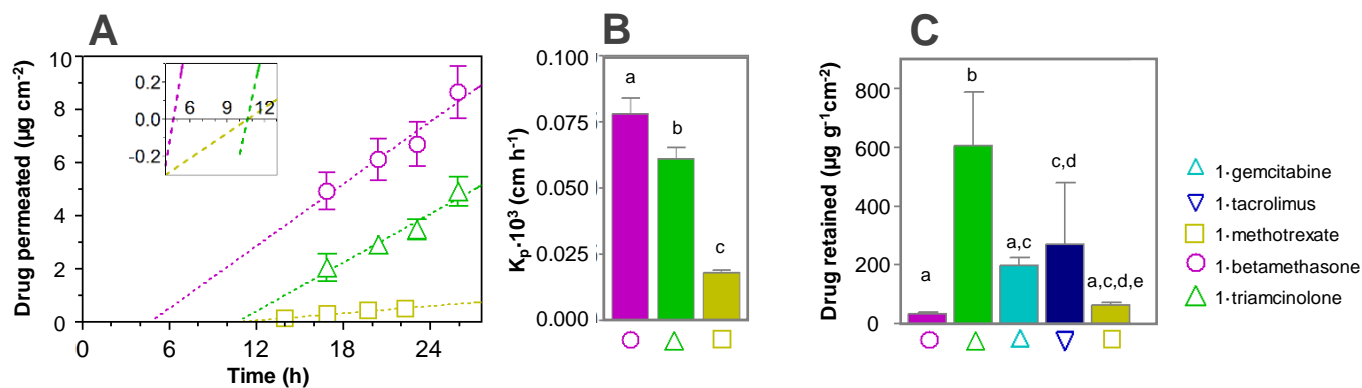
Figure 6. (A) Cumulative amount of drug permeated across human skin along time, after application of either **1·betamethasone**, **1·triamcinolone** or **1·methotrexate** gel; no drug permeation was observed after application of gels **1·gemcitabine** or **1·tacrolimus**. Values represent Means±SD (n=5). A linear least squares regression was performed. A magnification showing the T_{lag} (X-intercept) is shown inside the frame. (B) A comparison of the $K_p \cdot 10^3$ values calculated from drug permeation parameters. (C) Total amount of drug retained beneath the skin ($\mu g \, g^{-1} \, cm^{-2}$) after application of gels **1·betamethasone, 1·triamcinolone, 1·gemcitabine, 1·tacrolimus** or **1·methotrexate**, calculated according to the drug extraction and recovery experiments. Values represent Means ±SD (n=5). (B-C) Different lowercase letters represent significant differences (P<0.05).

13   Gel **1·betamethasone** showed the shortest lag-time (T_{lag}) (4.6 h), indicated by the time-axis intercept value

14   (Figure 6, A), and representing the time needed before the drug completely permeates the skin into the

15   receptor chamber, while both **1·triamcinolone** and **1·methotrexate** presented a similar T_{lag} (10.6 h) (See

16   Supporting Information, Table S 3).

17   The speed of drug permeation at the steady-state (transdermal flux), is calculated with the slope of the linear

18   regression [J_{SS}].[57] However, it is well known that the transdermal flux is dependent on the drug

19   concentration in the donor compartment (in the formulation applied) [C_0]. As gel **1·methotrexate** has a drug

20   concentration different than the other two gels, the permeability coefficient [K_p] (cm h^{-1}) was used as a

21   suitable parameter for comparing the permeation from all the different formulations, as it is obtained by

22   dividing the flux into C_0. For instance, it can be seen that the K_p of **1·betamethasone** is 28% higher than that

23   of **1·triamcinolone** ($0.06 \cdot 10^{-3} \pm 0.01 \cdot 10^{-3}$ cm h^{-1} and $0.08 \cdot 10^{-3} \pm 0.01 \cdot 10^{-3}$ cm h^{-1}respectively) (P<0.05), which

24   is also in accordance with previously reported results [26]. On the other hand, the K_p of **1·methotrexate** is

25   four times slower than the other two gels ($0.018 \cdot 10^{-3} \pm 0.002 \cdot 10^{-3}$ cm h^{-1}) (P<0.001). A graphic comparison

26   of K_p values can be seen in (Figure 6, B), and statistical analysis of K_p values is shown in Supporting

27   Information (Table S 4).

28   Considering the cytotoxicity of methotrexate as the intrinsic pharmacological mechanism, the possible

29   systemic toxicity that the application of the gel **1·methotrexate** may have, was determined by means of the

concentration in plasma at steady state (C_{ss}). It has been previously reported that mild toxicity might be manifested only at concentrations in serum above 10^{-6} M [58]. In this case, when **1·methotrexate** is applied on the skin, the maximum concentration in plasma (C_{ss}) that could be reached is around 0.086 µg/L (equivalent to 10^{-10} M), which is four-orders below toxic concentration, indicating that its topical application should not cause any undesired effects in other regions of the body. Cytotoxicity-calculation parameters are shown in Supporting Information (Table S 5).

The amount of drug remaining inside the skin can be calculated by means of both extraction and recovery assays. The extraction assay is performed to quantify the amount of drug that can be extracted from the skin after the experiment, while the recovery assay is performed to determine the experimental yield of the extraction, that is to say, the fraction of drug that can be extracted out of the total amount of drug that is actually retained. Results in Figure 6, (C), show that when incorporated in gel **1·2Br**, the five drugs under study are retained within the epidermis and outer dermis layers, which is optimum for the treatment of skin diseases such as Psoriasis. Percentage of recovery (recovery factor) and total amount of drug retained inside the skin, as well as the statistical analysis from these data, can be seen in Supporting Information (Table S 6 and Table S 7). For instance, the highest retention was seen with **1·triamcinolone** (605.4 ± 182.5 µg g^{-1} cm^{-2}), significantly higher than the rest of the gels (P<0.01 to P<0.001). On the other hand, the lowest retention was obtained with **1·betamethasone**, (31.6 ± 7.9 µg g^{-1} cm^{-2}). The high affinity of triamcinolone acetonide for the skin tissues is also reflected in the low percentage of recovery (9%) as compared to the rest of the drugs (above 27%).

Drug retention with **1·methotrexate** and **1·gemcitabine** was slightly higher than that from **1·betamethasone** (P<0.05), while with **1·tacrolimus** was significantly higher than that from **1·betamethasone**. As it can be seen, despite neither tacrolimus nor gemcitabine completely crossed the skin, both of them were able to penetrate the *stratum corneum* and keep retained inside the skin.

In-vivo studies

Several *in vivo* models have been developed to mimic the human pathology of Psoriasis, from complex immunological or genetic ones, to simple models reached by the repeated application of a substance such as imiquimod or 12-O-Tetradecanoylphorbol 13-acetate (TPA).[59–63] In this work, the repeated application of TPA along 10 days was used to induce the characteristic inflammation and hyperplasia seen in psoriatic skin [61], and the suitable gels were chosen for *in vivo* efficacy evaluation according to the results from drug release and skin permeation experiments. For instance, gels **1·betamethasone** or **1·triamcinolone** or **1·tacrolimus** or **1·methotrexate** or **1·2Br** (containing no drug), were applied in groups of mice to evaluate their anti-hyperplasia and anti-inflammatory activity. As well, drugs in solution, and the cream Celecrem®

containing betamethasone 17-valerate, as a reference marketed product, were applied for comparison purposes. The gel **1·gemcitabine** was not studied *in vivo* as it showed the lowest drug retention beneath the skin and it did not show permeation. A group of mice with daily TPA but without further treatment was taken as Control (psoriatic skin). The increase in ear thickness was plotted relative to the increase observed in psoriatic skin (Figure 7), and the main signs at the macroscopic level were observed (Figure 8).

As it can be seen by comparing healthy and psoriatic skin values, the daily TPA application leads to a gradual and significant increase in ear thickness (P<0.001) (Figure 7, A-F), and a gradual occurrence of roughness, desquamation, hair loss, and change of skin color, confirming the induced psoriasiform hyperplasia through the *in-vivo* model applied (Figure 8, B).

Gel **1·2Br** (without drug) shows to be majorly innocuous on healthy skin, as after 10-day application, it does not induce an increase in thickness (Figure 7, A), though a moderate exudate could be observed in tissue at day 11 (Figure 8, C). On the other hand, gel **1·2Br** without any drug, decreases on 25% the hyperplasia induced by TPA application, though it cannot be considered a significant difference (P<0.05) (Figure 7, A), and the signs observed do not represent an improvement as compared to psoriatic skin (Figure 8, D).
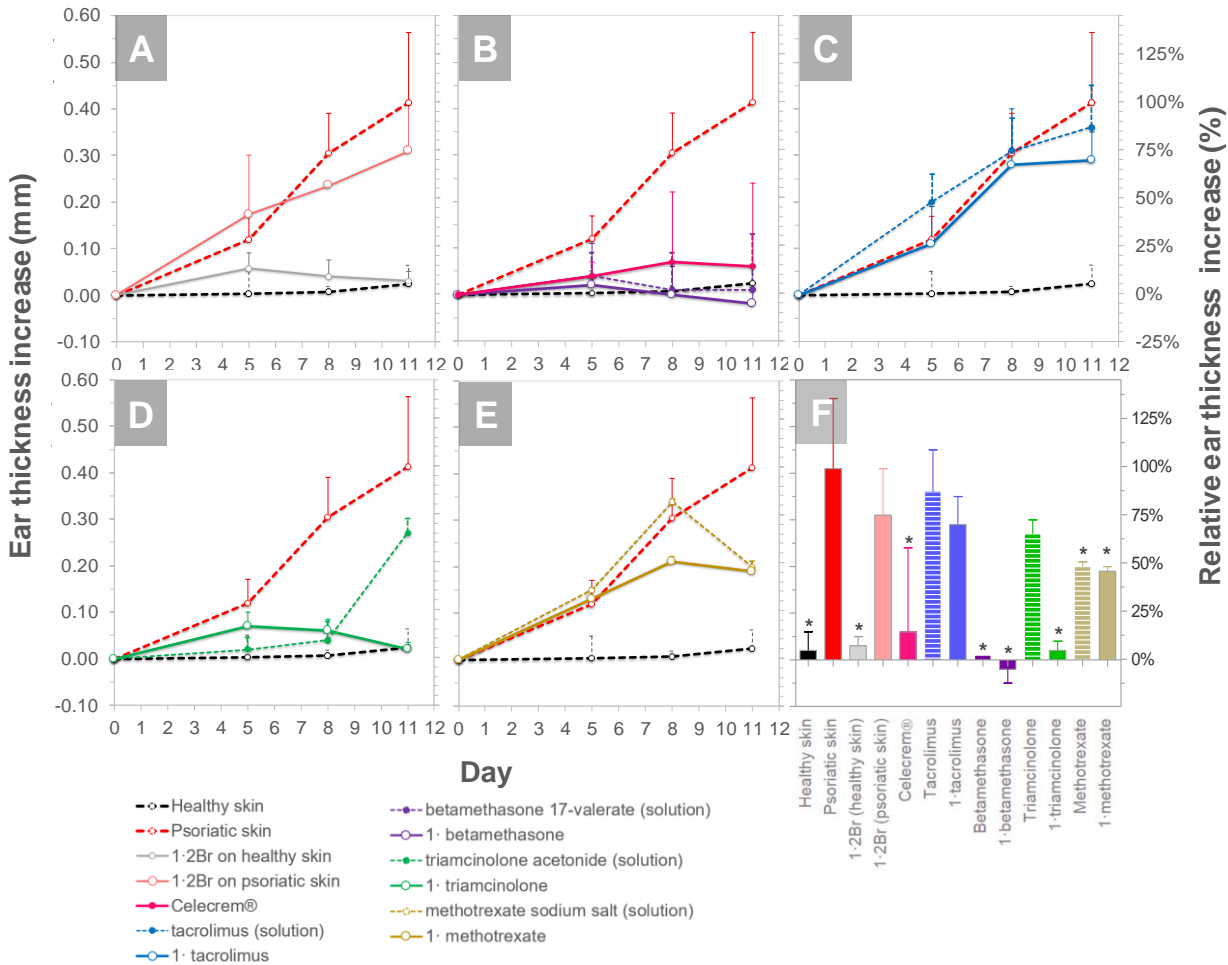
Figure 7. Ear thickness increase (both in absolute values [mm/left axis] and relative to psoriatic skin [%/right axis]) of mice along 10-day application of TPA and a treatment. (A-F) TPA application without further treatment was taken as psoriatic skin, while ears with neither TPA nor treatment were taken as healthy skin. (A) **1·2Br** (both on healthy and psoriatic skin). (B) Betamethasone 17-valerate solution, gel **1·betamethasone**, and Celecrem® (containing betamethasone 17-valerate). (C) Tacrolimus solution and gel **1·tacrolimus**. (D) Triamcinolone acetonide solution and gel **1·triamcinolone**. (E) Methotrexate sodium salt solution and gel **1·methotrexate**. (F) Ear thickness increase at day 11 (overall view). Values represent Means±SD (n=6). *Significant difference versus psoriatic skin (P<0.05).

Group	Day 5	Day 8	Day 11	Observations
A Control (-) (Healthy skin)				Thickness uniformity, no desquamation, no roughness, no rigidity, no color change, no hair loss, no blood marks, no tissue vasculature alterations, no exudates.
B Control (+) (Psoriatic skin)				Thickness increase, desquamation, roughness, color change, hair loss, blood marks.
C 1·2Br (in healthy skin)				Very slight increase in thickness and roughness. Moderate exudate at day 11.
D 1·2Br (in psoriatic skin)				Desquamation, color change, tissue damage in some areas.
E Celecrem® (betamethasone 17-valerate)				Tissue vasculature altered on days 5 and 8, but restored on day 11.
F Betamethasone 17-valerate (solution)				Tissue vasculature slightly altered.
G 1·betamethasone				Tissue vasculature slightly altered.
H Triamcinolone acetonide (solution)				Tissue vasculature slightly altered from day 5. Hair loss and increase in thickness and rigidity on day 11.
I 1·triamcinolone				Moderate rigidity.
J Tacrolimus (solution)				Moderate hair loss, moderate desquamation.
K 1·tacrolimus				Tissue vasculature moderately altered and blood marks.
L Methotrexate sodium salt (solution)				Increase in thickness and rigidity from day 5, hair loss, exudate from day 8.
M 1·methotrexate				Rigidity from day 8.

The commercially available Celecrem®, containing betamethasone 17-valerate, was tested as the reference product for comparison purposes, which showed a significant 85% decrease in ear thickness ($P<0.001$) (Figure 7, B), though it could not prevent the vasculature alterations and blood marks up to day 8, but it restored the normal vasculature at day 11 (Figure 8, E), confirming the efficacy of the commercial treatment. Similarly, the application of betamethasone 17-valerate in acetone solution showed a 95% thickness decrease, also showing vasculature alterations at the macroscopic level (Figure 8, F), while the application of betamethasone 17-valerate in gel (**1·betamethasone**), lead to the highest efficacy showing thickness decrease even below the basal values (Figure 7, B). Though no significant difference is observed as compared to the commercial product, this slightly higher efficacy might be related to the higher permeation and higher retention observed from gel **1·betamethasone** as compared to the marketed product [26].

Similar increased efficacies can be observed when other drugs are incorporated in gel **1·2Br**. For instance, the drug tacrolimus in solution did not show a significant thickness decrease (9%); however, when it is incorporated in gel (**1·tacrolimus**), a 50% decrease can be observed ($P<0.05$) (Figure 7, C). This is in accordance with the macroscopic observations, where the mice treated with tacrolimus in solution gradually showed hair loss and the desquamation observed in psoriatic skin (Figure 8, J), while the mice treated with gel **1·tacrolimus** showed moderately altered vasculature but no hair loss and desquamation (Figure 8, K).

As well, the application of triamcinolone acetonide in solution, which is also corticoid drug, only lead to a 35% thickness decrease ($P>0.05$); nonetheless, the treatment with gel **1·triamcinolone** results in 95% decrease ($P<0.001$) (Figure 7, D). Similarly, the macroscopic observations of mice treated with triamcinolone acetonide in solution show some vasculature alterations, ear rigidity, and a complete hair loss at day 11 (Figure 8, H), while the mice treated with gel **1·triamcinolone** only show rigidity but no hair loss (Figure 8, I). These results also correlate with the skin permeation and retention experiments, where tacrolimus and both corticoid drugs triamcinolone acetonide and betamethasone 17-valerate, after topical application, are highly retained inside the skin, where they perform their therapeutic activity.

Finally, the treatment with methotrexate sodium salt solution appears to decrease in 50% the ear thickness only at day 11, while the application of gel **1·methotrexate** shows a similar decrease already from day 8, and which is maintained until day 11 (Figure 7, D), suggesting a promoted drug permeation or retention inside the skin by the supramolecular gel. This increased efficacy can also be seen at the macroscopic tissue observations as the application of the drug in solution lead to rigidity, complete hair loss and exudate from day 8, while the application of gel **1·methotrexate** induced ear rigidity, but neither hair loss nor exudate.

1 CONCLUSION

2   In this work, the gelling ability of a dicationic bis-imidazolium amphiphile **1·2Br** has been proven to be
3 effective when structurally diverse drugs, including compounds with different charge and lipophilicity, and
4 which are used in the treatment of Psoriasis, were incorporated into the mixture. Hydrogels can be
5 spontaneously formed in only 10 min in presence of only ethanol and water. A nucleation enhancement of
6 the gelation process was observed with low concentrations of either methotrexate sodium salt or tacrolimus
7 or triamcinolone acetonide, as gel formation time is decreased; however, the further increase in drug
8 concentration increases gelation time. The fibrous structure of the gels at the nanoscale is influenced by the
9 drug included. Interestingly, when the gel is formed in presence of gemcitabine hydrochloride, no apparent
10 drug precipitates can be found in xerogels as observed by SEM, suggesting a nanoscaled precipitation, which
11 is optimum for biopharmaceutical purposes. The structural differences of the xerogels can be also correlated
12 with their viscoelastic properties, where the gel showing more network entanglement (**1·methotrexate**)
13 presented both the highest resistances to rupture and to deformation. Nonetheless, all the formulations could
14 be categorized as soft solid-like gels, which can break abruptly when critical stress is reached, and therefore
15 optimum for application over irritated skin such as that in Psoriasis. Gels can incorporate drugs not only
16 dissolved at the interstitial space, but also 72% of either gemcitabine or methotrexate, and 38% of tacrolimus,
17 are found within their fibers when gels are formed at a 1:1 molar ratio. This unique fiber-incorporation
18 behavior acts as a packaging that protects the drug, and importantly, influences a *Two-Phase Exponential*
19 drug release profile, in which the first phase corresponds to the drug dissolved in the interstitial space of the
20 gel, and the second phase corresponds to the drug exiting from the fibers. The release kinetics of each phase
21 is related with the physicochemical properties of the drugs, where the speed in the first phase is promoted by
22 low or moderate log P values and moderate or high water solubilities, while the speed in the second phase is
23 promoted by moderate to high log P values but also moderate to high water solubilities. As a result, highly
24 hydrophilic drugs such as gemcitabine hydrochloride show a fast release in the first phase but a slow sustained
25 release in the second phase; highly hydrophobic drugs such as tacrolimus are released at a moderate speed in
26 both phases; whereas drugs with moderate hydrophobicity such as betamethasone 17-valerate and
27 triamcinolone acetonide are rapidly released in both phases.)

28   Moreover, skin permeation tests show the complete permeation of betamethasone 17-valerate,
29 triamcinolone acetonide and methotrexate, while all the drugs (including gemcitabine and tacrolimus), after
30 application, are retained inside the skin, where they perform they therapeutic activity. In addition, the
31 application of **1·methotrexate** leads to drug concentrations in plasma fairly below the toxic ones, for which
32 the application of this gel can be considered safe for other parts of the body. Finally, *in vivo* studies show
33 that the application of these gels incorporating a drug successfully prevent the typical hyperplasia and tissue
34 degeneration observed in Psoriasis ($P<0.01$), especially in the cases of **1·triamcinolone** and

1·betamethasone, which completely prevent the skin thickness increase. These two gels also show slightly improved efficacies as compared to the commercial product Celecrem® containing betamethasone 17-valerate. Moreover, the incorporation of the drugs in these gels seems to promote the efficacy as compared to the drugs in acetone solution, both at the anti-hyperplasia activity and at preventing tissue degeneration, a behavior that could be related to the retention of the drug beneath the skin promoted by the gel.

The topical application of these gels shows to be an interesting option in the treatment of skin diseases such as Psoriasis, avoiding the first pass metabolism associated with oral dosages, and increasing the drug concentration at the pharmacological target, while their great versatility and ease of preparation provides great scalability for industrial commercialization.

SUPPORTING INFORMATION AVAILABLE

Frequency sweep tests (Figure S 1), ^{1}H-NMR spectra from drug incorporation studies (Figure S 2 - Figure S 4), SEM images of drug precipites previously in solution (Figure S 5), Drug release parameters (Table S 1), Physicochemical properties of drugs under study (Table S 2), Skin permeation parameters (Table S 3), Statistical analysis of K_p values (Table S 4), Evaluation of **1·methotrexate** systemic toxicity (Table S 5), Drug retention inside the skin with statistical analysis (Table S 6 and Table S 7), and statistical analysis of *in vivo* experiments (Table S 8), are available and can be found in Supporting Information.

ACKNOWLEDGEMENTS

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Nanostructured supramolecular hydrogels: towards the topical treatment of Psoriasis and other skin diseases

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Rheological studies

Figure S 1. Elastic modulus (G') and loss modulus (G'') as a function of stress frequency for samples: (A) **1·2Br** (B) **1·gemcitabine**, (C) **1·tacrolimus** and (D) **1·methotrexate**.

Drug incorporation studies
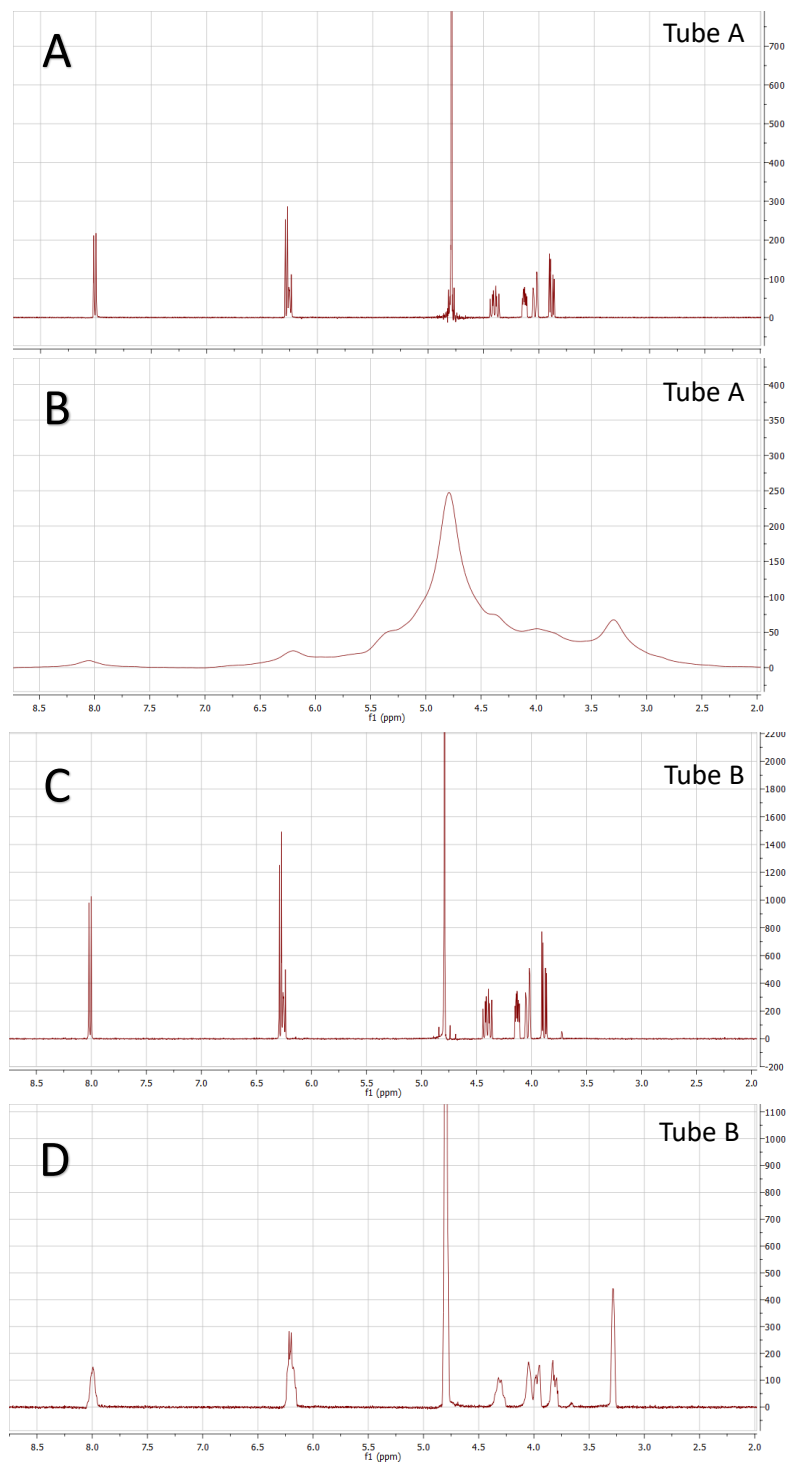
Figure S 2. ^{1}H NMR spectra corresponding to the incorporation of gemcitabine hydrochloride within the gel.
A) Tube A, Record 1: gemcitabine hydrochloride (8.28 μmol) dissolved in 0.75 mL of deuterium oxide.
B) Tube A, Record 2: after addition of **1·2Br** (8.28 μmol) in 0.75 mL of deuterated methanol.
C) Tube B, Record 1: gemcitabine hydrochloride (8.28 μmol) dissolved in 0.75 mL of deuterium oxide.
D) Tube B, Record 2: after addition of 0.75 mL of deuterated methanol.
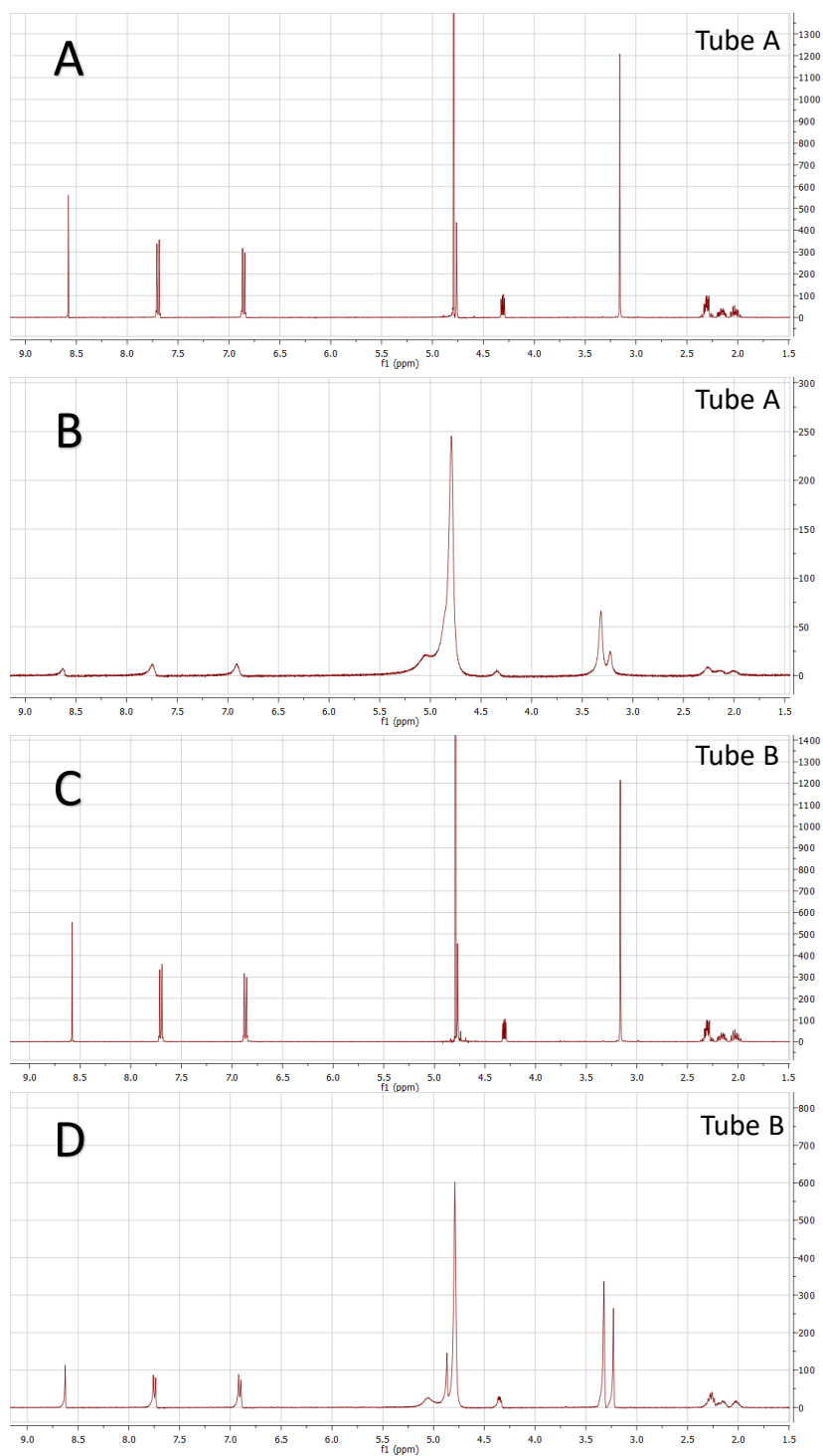
Figure S 3. . ^{1}H NMR spectra corresponding to the incorporation of methotrexate sodium salt within the gel.
A) Tube A, Record 1: Methotrexate sodium salt (8.28 μmol) dissolved in 0.75 mL of deuterium oxide.
B) Tube A, Record 2: after addition of **1·2Br** (8.28 μmol) in 0.75 mL of deuterated methanol.
C) Tube B, Record 1: Methotrexate sodium salt (8.28 μmol) dissolved in 0.75 mL of deuterium oxide.
D) Tube B, Record 2: after addition of 0.75 mL of deuterated methanol.
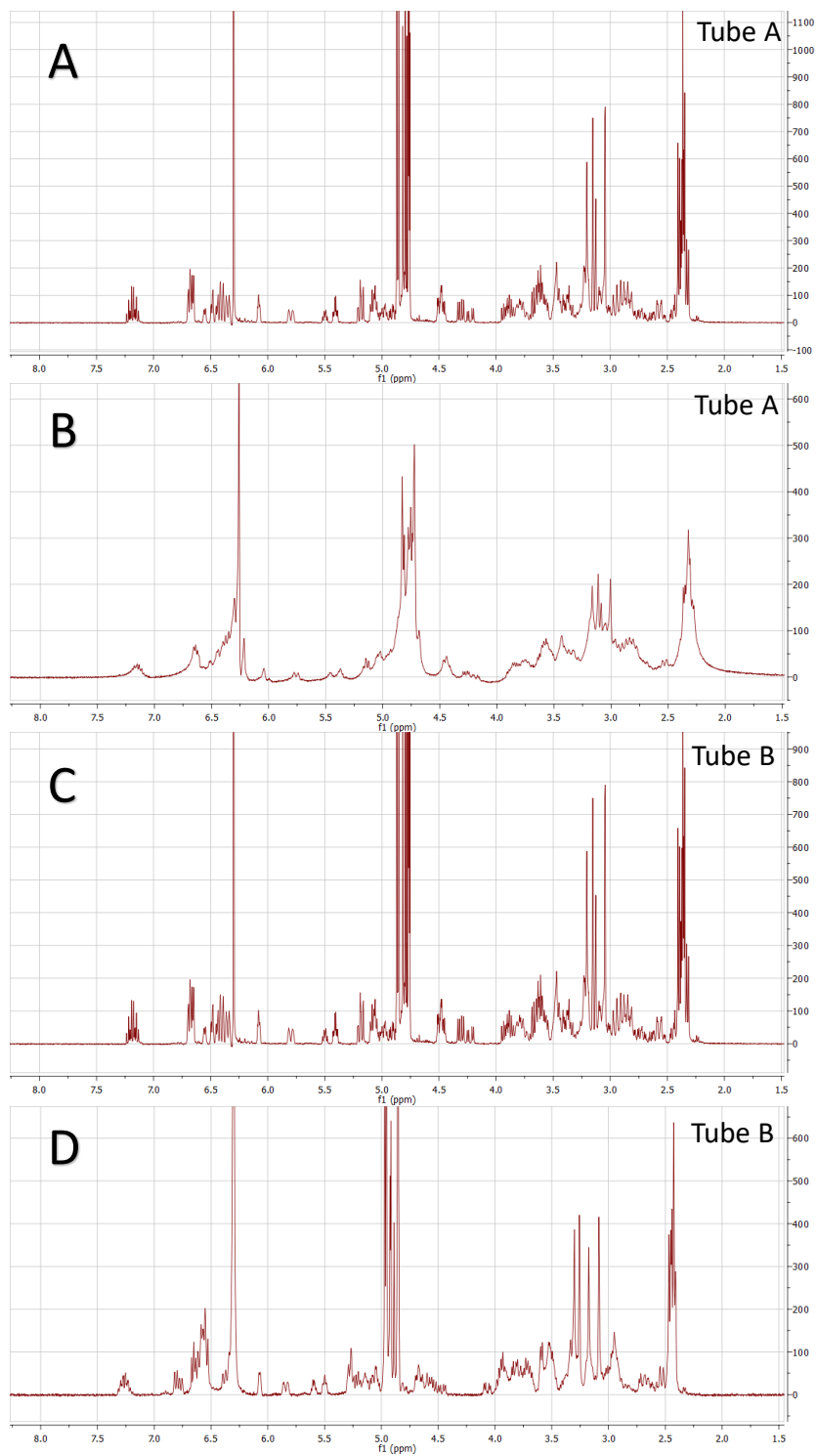
Figure S 4. ^{1}H NMR spectra corresponding to the incorporation of tacrolimus within the gel.
A) Tube A, Record 1: tacrolimus (8.28 μmol) dissolved in 0.75 mL of deuterated methanol.
B) Tube A, Record 2: after dissolving **1·2Br** (8.28 μmol) in Tube A and adding 0.75 mL of deuterium oxide.
C) Tube B, Record 1: tacrolimus (8.28 μmol) dissolved in 0.75 mL of deuterated methanol.
D) Tube B, Record 2: after addition of 0.75 mL of deuterium oxide.

Microscopy
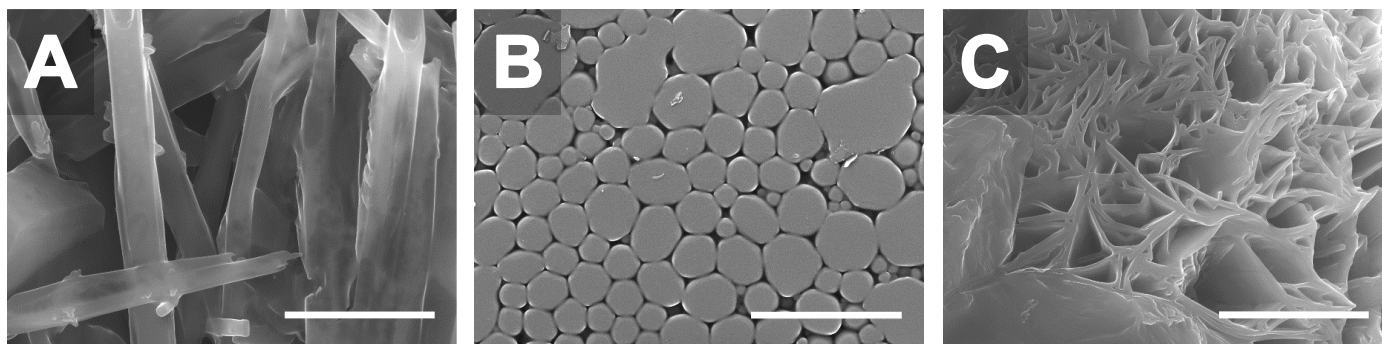
Figure S 5. SEM images of drug precipitates of gemcitabine hydrochloride (A), tacrolimus (B), and methotrexate sodium salt (C), previously in a 2.5 mg/mL solution in ethanol:water (50:50) without gelator. Scalebar represents 5 µm.

Drug release studies

Table S 1. Drug release parameters from gels under study.

	1·gemcitabine	1·tacrolimus	1·methotrexate	1·triamcinolone[a]		1·betamethasone[a]	
Model	Two Phase Exponential Association	Two Phase Exponential Association	Two Phase Exponential Association	One Phase Exponential Association	Two Phase Exponential Association	One Phase Exponential Association	Two Phase Exponential Association
Y_{MAX1} (%)	59.1 ± 0.9	31.0 ± 48.3	41.4 ± 1.7	90.9 ± 1.60	74	79.2 ± 3.14	66
K1 (h^{-1})	0.4 ± 0.01	0.1 ± 0.12	0.4 ± 0.04	0.2 ± 0.02	0.25	0.3 ± 0.04	0.3
Y_{MAX2} (%)	209.2 ± 214.6	209.7 ± 186.4	185.4 ± 163.0		17		13
K2 (h^{-1})	0.0002 ± 0.0220	0.0011 ± 0.1116	0.0010 ± 0.0094		0.25		0.3
R^2	0.9999	0.9918	0.9994	0.9965	0.9964	0.9832	0.9816

aData taken from [26]

Drug physicochemical properties

Table S 2. Physicochemical properties of drugs betamethasone 17-valerate, triamcinolone acetonide, gemcitabine, tacrolimus and methotrexate sodium salt.

Drug	Log P	MW	Solubility in water (mg/L)	Ref.
Betamethasone 17-valerate	3.57	476.6	9.3	[64,65]
Triamcinolone acetonide	2.5	434.5	20.9	[66]
Tacrolimus	3.19 to 5.59	804.0	$1.82e^{-2}$	[53,67,68]
Gemcitabine	-1.4 to -1.24	263.2	$5.13e^4$	[69–71]
Methotrexate sodium salt	-1.85 to -0.5	454.4	$2.6e^3$	[72–74]

Skin permeation studies

1) Skin permeation parameters

Table S 3. Skin permeation parameters from drugs upon application of gels **1·betamethasone**, **1·triamcinolone** and **1·methotrexate** on human skin. Values represent the calculated parameters ± SD (n=5).

Parameters	1·betamethasone	1·triamcinolone	1·methotrexate
J_{ss} ($\mu g\ cm^{-2}\ h^{-1}$)	0.391±0.066	0.306±0.047	0.045±0.005
T_{lag} (h)	4.64	10.61	10.60
C_0 ($mg\ cm^{-3}$)	5	5	2.5
$Kp \cdot 10^3$ ($cm\ h^{-1}$)	0.078±0.013	0.061±0.009	0.018±0.002

Table S 4. One-way Analysis of Variance with Tukey's multiple comparison test of K_p values obtained from skin permeation of gels **1·betamethasone**, **1·triamcinolone** and **1·methotrexate.**

Parameter	Value		
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	3		
F	53,79		
R squared	0,8997		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	8,343		
P value	0,0154		
P value summary	*		
Do the variances differ signif. (P < 0.05)	Yes		
ANOVA Table	SS	df	MS
Treatment (between columns)	0,009571	2	0,004786
Residual (within columns)	0,001068	12	0,00008896
Total	0,01064	14	
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
1·betamethasone vs 1·triamcinolone	0,01702	4,035	P < 0.05	0.001105 to 0.03294
1·betamethasone vs 1·methotrexate	0,06003	14,23	P < 0.001	0.04411 to 0.07594
1·triamcinolone vs 1·methotrexate	0,04301	10,20	P < 0.001	0.02709 to 0.05892

2) Prediction of systemic toxicity of gel 1·methotrexate

A possible systemic toxicity from the topical application of **1·methotrexe** was estimated by means of the plasmatic drug concentration at steady state (C_{ss}). C_{ss} is calculated by means of the following equation:

$$C_{SS} = \frac{J_{SS} \cdot SAT_{area}}{CL_P}$$

where SAT_{area} is the hypothetical area of topical application (e.g. 10 cm^2) and CL_p corresponds to the methotrexate sodium salt plasmatic clearance. It is reported that methotrexate sodium salt plasmatic clearance presents a strong variability,[58,75–78] so an indicative median value of 5.5 L h^{-1} was used.

Table S 5. Calculated methotrexate plasma concentration at steady state (Css) upon topical application of gel **1·methotrexate**.and determination of the systemic toxicity. [a]Hypothetical area for dermal application. [b]Plasmatic clearance of methotrexate is reported to have a great variability. A median value of 5.5 L h^{-1} is taken. [c]Reported plasmatic concentration that shows systemic toxicity.

	1·methotrexate
Css (µg L^{-1})	0.086
SAT$_{area}$ (cm^2)[a]	10
CL$_P$ (L h^{-1})[b]	5.5
Toxicity systemic concentration (M)[c]	3·10^{-6}
Toxicity systemic concentration (µg cm^{-3})[c]	1.36

3) Drug retention inside the skin

By means of both extraction and recovery experimental results, the real amount of drug that remained inside the skin after the permeation studies can be calculated

$$Q_{ret} = \frac{Q_{ext}}{(R \cdot W_{skin} \cdot A_{per})}$$

where Q_{ret} is quantity of drug retained into the skin, Q_{ext} is the amount that was possible to be extracted from the skin, R is the recovery factor (the fraction of drug that can be extracted out of the total amount of drug that is actually retained), A_{per} is the area of permeation (2.54 cm^2) and W_{skin} corresponds to the weight of each skin permeated section.

Table S 6. Drug recovery factors and calculated amount of drug retained inside the skin after permeation experiments with gels **1·betamethasone, 1·triamcinolone, 1·gemcitabine, 1·tacrolimus** and **1·methotrexate.** Drug retained is expressed in µg of drug per gram and square centimeter of skin ± SD.

Drug	Recovery factor (R)	Drug retained ($\mu g\ g^{-1}\ cm^{-2}$)
Betamethasone 17-valerate	0.27	31.6 ± 7.9
Triamcinolone acetonide	0.09	605.4 ± 182.5
Gemcitabine	0.31	198.1 ± 27.9
Tacrolimus	0.36	269.4 ± 211.2
Methotrexate	0.28	61.5 ± 11.1

Table S 7. One-way ANOVA with Tukey's multiple comparison test from the amount of drug retained values.

One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	16,73			
R squared	0,7700			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	42,19			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			

ANOVA Table	SS	df	MS	
Treatment (between columns)	1056000	4	264000	
Residual (within columns)	315600	20	15780	
Total	1372000	24		

Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
1·betamethasone vs 1·triamcinolone	-573,8	10,21	P < 0.001	-811.6 to -336.1
1·betamethasone vs 1·gemcitabine	-166,5	2,965	P > 0.05	-404.3 to 71.20
1·betamethasone vs 1·tacrolimus	-238,2	4,240	P < 0.05	-475.9 to -0.4704
1·betamethasone vs 1·methotrexate	-29,91	0,5324	P > 0.05	-267.6 to 207.8
1·triamcinolone vs 1·gemcitabine	407,3	7,250	P < 0.001	169.5 to 645.0
1·triamcinolone vs 1·tacrolimus	335,6	5,974	P < 0.01	97.88 to 573.3
1·triamcinolone vs 1·methotrexate	543,9	9,682	P < 0.001	306.2 to 781.6
1·gemcitabine vs 1·tacrolimus	-71,67	1,276	P > 0.05	-309.4 to 166.1
1·gemcitabine vs 1·methotrexate	136,6	2,432	P > 0.05	-101.1 to 374.4
1·tacrolimus vs 1·methotrexate	208,3	3,708	P > 0.05	-29.44 to 446.0

In-vivo experiments

Table S 8. One-way ANOVA with Tukey's multiple comparison test from the ear thickness increase values at day 11 after TPA and treatment application.

One-way analysis of variance			
P value	< 0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	13		
F	17,47		
R squared	0,7718		
ANOVA Table	SS	df	MS
Treatment (between columns)	1,597	12	0,1331
Residual (within columns)	0,4722	62	0,007616
Total	2,069	74	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Healthy skin (Control(-)) vs Psoriatic skin (Control(+))	-0,3900	8,938	Yes	***	-0.6027 to -0.1773
Healthy skin (Control(-)) vs **1·2Br** (healthy skin)	-0,0100	0,2292	No	ns	-0.2227 to 0.2027
Healthy skin (Control(-)) vs **1·2Br** (psoriatic skin)	-0,2900	6,646	Yes	**	-0.5027 to -0.07729
Healthy skin (Control(-)) vs Celecrem®	-0,0400	0,9167	No	ns	-0.2527 to 0.1727
Healthy skin (Control(-)) vs Tacrolimus	-0,3400	7,792	Yes	***	-0.5527 to -0.1273
Healthy skin (Control(-)) vs **1·tacrolimus**	-0,2700	6,188	Yes	**	-0.4827 to -0.05729
Healthy skin (Control(-)) vs Betamethasone	0,0100	0,2292	No	ns	-0.2027 to 0.2227
Healthy skin (Control(-)) vs **1·betamethasone**	0,0400	0,9167	No	ns	-0.1727 to 0.2527
Healthy skin (Control(-)) vs Triamcinolone	-0,2500	5,729	Yes	**	-0.4627 to -0.03729
Healthy skin (Control(-)) vs **1·triamcinolone**	0,0000	0,0000	No	ns	-0.2127 to 0.2127
Healthy skin (Control(-)) vs Methotrexate	-0,1800	4,125	No	ns	-0.3927 to 0.03271
Healthy skin (Control(-)) vs **1·methotrexate**	-0,1700	3,896	No	ns	-0.3827 to 0.04271
Psoriatic skin (Control(+)) vs **1·2Br** (healthy skin)	0,3800	10,67	Yes	***	0.2063 to 0.5537
Psoriatic skin (Control(+)) vs **1·2Br** (psoriatic skin)	0,1000	2,807	No	ns	-0.07368 to 0.2737
Psoriatic skin (Control(+)) vs Celecrem®	0,3500	9,824	Yes	***	0.1763 to 0.5237
Psoriatic skin (Control(+)) vs Tacrolimus	0,05000	1,403	No	ns	-0.1237 to 0.2237
Psoriatic skin (Control(+)) vs **1·tacrolimus**	0,1200	3,368	No	ns	-0.05368 to 0.2937
Psoriatic skin (Control(+)) vs Betamethasone	0,4000	11,23	Yes	***	0.2263 to 0.5737
Psoriatic skin (Control(+)) vs **1·betamethasone**	0,4300	12,07	Yes	***	0.2563 to 0.6037
Psoriatic skin (Control(+)) vs Triamcinolone	0,1400	3,929	No	ns	-0.03368 to 0.3137
Psoriatic skin (Control(+)) vs **1·triamcinolone**	0,3900	10,95	Yes	***	0.2163 to 0.5637
Psoriatic skin (Control(+)) vs Methotrexate	0,2100	5,894	Yes	**	0.03632 to 0.3837
Psoriatic skin (Control(+)) vs **1·methotrexate**	0,2200	6,175	Yes	**	0.04632 to 0.3937
1·2Br (healthy skin) vs **1·2Br** (psoriatic skin)	-0,2800	7,859	Yes	***	-0.4537 to -0.1063
1·2Br (healthy skin) vs Celecrem®	-0,0300	0,8420	No	ns	-0.2037 to 0.1437
1·2Br (healthy skin) vs Tacrolimus	-0,3300	9,262	Yes	***	-0.5037 to -0.1563
1·2Br (healthy skin) vs **1·tacrolimus**	-0,2600	7,298	Yes	***	-0.4337 to -0.08632
1·2Br (healthy skin) vs Betamethasone	0,0200	0,5614	No	ns	-0.1537 to 0.1937
1·2Br (healthy skin) vs 1·betamethasone	0,0500	1,403	No	ns	-0.1237 to 0.2237
1·2Br (healthy skin) vs Triamcinolone	-0,2400	6,736	Yes	***	-0.4137 to -0.06632
1·2Br (healthy skin) vs 1·triamcinolone	0,0100	0,2807	No	ns	-0.1637 to 0.1837
1·2Br (healthy skin) vs Methotrexate	-0,1700	4,772	No	ns	-0.3437 to 0.003678
1·2Br (healthy skin) vs **1·methotrexate**	-0,1600	4,491	No	ns	-0.3337 to 0.01368
1·2Br (psoriatic skin) vs Celecrem®	0,2500	7,017	Yes	***	0.07632 to 0.4237
1·2Br (psoriatic skin) vs Tacrolimus	-0,05000	1,403	No	ns	-0.2237 to 0.1237
1·2Br (psoriatic skin) vs **1·tacrolimus**	0,02000	0,5614	No	ns	-0.1537 to 0.1937
1·2Br (psoriatic skin) vs Betamethasone	0,3000	8,420	Yes	***	0.1263 to 0.4737
1·2Br (psoriatic skin) vs **1·betamethasone**	0,3300	9,262	Yes	***	0.1563 to 0.5037

1·2Br (psoriatic skin) vs Triamcinolone	0,04000	1,123	No	ns	-0.1337 to 0.2137
1·2Br (psoriatic skin) vs **1·triamcinolone**	0,2900	8,140	Yes	***	0.1163 to 0.4637
1·2Br (psoriatic skin) vs Methotrexate	0,1100	3,087	No	ns	-0.06368 to 0.2837
1·2Br (psoriatic skin) vs **1·methotrexate**	0,1200	3,368	No	ns	-0.05368 to 0.2937
Celecrem® vs Tacrolimus	-0,3000	8,420	Yes	***	-0.4737 to -0.1263
Celecrem® vs **1·tacrolimus**	-0,2300	6,456	Yes	**	-0.4037 to -0.05632
Celecrem® vs Betamethasone	0,0500	1,403	No	ns	-0.1237 to 0.2237
Celecrem® vs 1·betamethasone	0,0800	2,245	No	ns	-0.09368 to 0.2537
Celecrem® vs Triamcinolone	-0,2100	5,894	Yes	**	-0.3837 to -0.03632
Celecrem® vs 1·triamcinolone	0,0400	1,123	No	ns	-0.1337 to 0.2137
Celecrem® vs Methotrexate	-0,1400	3,929	No	ns	-0.3137 to 0.03368
Celecrem® vs 1·methotrexate	-0,1300	3,649	No	ns	-0.3037 to 0.04368
Tacrolimus vs **1·tacrolimus**	0,07000	1,965	No	ns	-0.1037 to 0.2437
Tacrolimus vs Betamethasone	0,3500	9,824	Yes	***	0.1763 to 0.5237
Tacrolimus vs 1·betamethasone	0,3800	10,67	Yes	***	0.2063 to 0.5537
Tacrolimus vs Triamcinolone	0,0900	2,526	No	ns	-0.08368 to 0.2637
Tacrolimus vs 1·triamcinolone	0,3400	9,543	Yes	***	0.1663 to 0.5137
Tacrolimus vs Methotrexate	0,1600	4,491	No	ns	-0.01368 to 0.3337
Tacrolimus vs **1·methotrexate**	0,1700	4,772	No	ns	-0.003678 to 0.3437
1·tacrolimus vs Betamethasone	0,2800	7,859	Yes	***	0.1063 to 0.4537
1·tacrolimus vs 1·betamethasone	0,3100	8,701	Yes	***	0.1363 to 0.4837
1·tacrolimus vs Triamcinolone	0,02000	0,5614	No	ns	-0.1537 to 0.1937
1·tacrolimus vs 1·triamcinolone	0,2700	7,578	Yes	***	0.09632 to 0.4437
1·tacrolimus vs Methotrexate	0,09000	2,526	No	ns	-0.08368 to 0.2637
1·tacrolimus vs 1·methotrexate	0,1000	2,807	No	ns	-0.07368 to 0.2737
Betamethasone vs **1·betamethasone**	0,0300	0,8420	No	ns	-0.1437 to 0.2037
Betamethasone vs Triamcinolone	-0,2600	7,298	Yes	***	-0.4337 to -0.08632
Betamethasone vs **1·triamcinolone**	-0,0100	0,2807	No	ns	-0.1837 to 0.1637
Betamethasone vs Methotrexate	-0,1900	5,333	Yes	*	-0.3637 to -0.01632
Betamethasone vs **1·methotrexate**	-0,1800	5,052	Yes	*	-0.3537 to -0.006322
1·betamethasone vs Triamcinolone	-0,2900	8,140	Yes	***	-0.4637 to -0.1163
1·betamethasone vs 1·triamcinolone	-0,0400	1,123	No	ns	-0.2137 to 0.1337
1·betamethasone vs Methotrexate	-0,2200	6,175	Yes	**	-0.3937 to -0.04632
1·betamethasone vs 1·methotrexate	-0,2100	5,894	Yes	**	-0.3837 to -0.03632
Triamcinolone vs **1·triamcinolone**	0,2500	7,017	Yes	***	0.07632 to 0.4237
Triamcinolone vs Methotrexate	0,07000	1,965	No	ns	-0.1037 to 0.2437
Triamcinolone vs **1·methotrexate**	0,08000	2,245	No	ns	-0.09368 to 0.2537
1·triamcinolone vs Methotrexate	-0,1800	5,052	Yes	*	-0.3537 to -0.006322
1·triamcinolone vs 1·methotrexate	-0,1700	4,772	No	ns	-0.3437 to 0.003678
Methotrexate vs **1·methotrexate**	0,01000	0,2807	No	ns	-0.1637 to 0.1837