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Improvement of Topical Palmitoylethanolamide Anti-Inflammatory Activity by Pegylated Prodrugs

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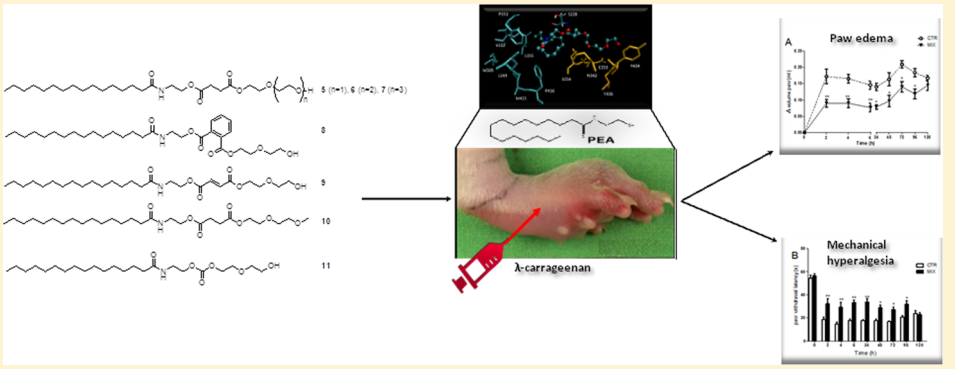

ABSTRACT: A small library of polyethylene glycol esters of palmitoylethanolamide (PEA) was synthesized with the aim of improving the pharmacokinetic profile of the parent drug after topical administration. Synthesized prodrugs were studied for their skin accumulation, pharmacological activities, in vitro chemical stability, and in silico enzymatic hydrolysis. Prodrugs proved to be able to delay and prolong the pharmacological activity of PEA by modification of its skin accumulation profile. Pharmacokinetic improvements were particularly evident when specific structural requirements, such as flexibility and reduced molecular weight, were respected. Some of the synthesized prodrugs prolonged the pharmacological effects 5 days following topical administration, while a formulation composed by PEA and two pegylated prodrugs showed both rapid onset and long-lasting activity, suggesting the potential use of polyethylene glycol prodrugs of PEA as a suitable candidate for the treatment of skin inflammatory diseases.

KEYWORDS: *N-palmitoylethanolamide, pegylated prodrugs, topical delivery, anti-inflammatory effects, antihyperalgesic effects*

INTRODUCTION

Fatty acid ethanolamides (FAEs) are a family of lipids that participate in the control of multiple physiological functions, including pain and inflammation.[1−3] Palmitoylethanolamide (PEA) is a member of this family, it has been widely reported that PEA exerts antinociceptive and anti-inflammatory effects in experimental animals and humans.[1,2,4] Such effects are primarily, albeit not exclusively,[1] due to the ability of PEA to bind with high affinity peroxisome proliferator-activated receptor (PPAR)-alpha.[2] The presence of PPAR-α has been demonstrated in rodent and human keratinocytes[5,6] as well in mouse and human dermis[7,8] and, in addition, in some specific cells such as sebocytes, cutaneous macrophages, and T-lymphocytes.[9−11]

Several data indicate that topically administered PEA formulations inhibit inflammation and reduce dermatitis with a potency comparable to the synthetic PPAR-α agonist, Wy-14643.[12−14] Moreover, PEA ointments have been proposed as an effective therapeutic tool in different skin disorders, both for human and veterinary use, such as dermatitis, pruritus, psoriasis, and skin barrier disruption.[15−18] Finally, it has been shown that topical application of PPAR-α activators limits their pharmacological activity to the skin, avoiding any systemic side effects.[12] However, the use of topical PEA is strongly limited by short-term activity. It is widely reported that repeated daily topical administration of PEA ointments are necessary to achieve remarkable pharmacological effects.[12,15−18] Thus, modification of pharmacokinetic properties of the molecule is required to gain a sustained and prolonged biological activity, as well as a better compliance.

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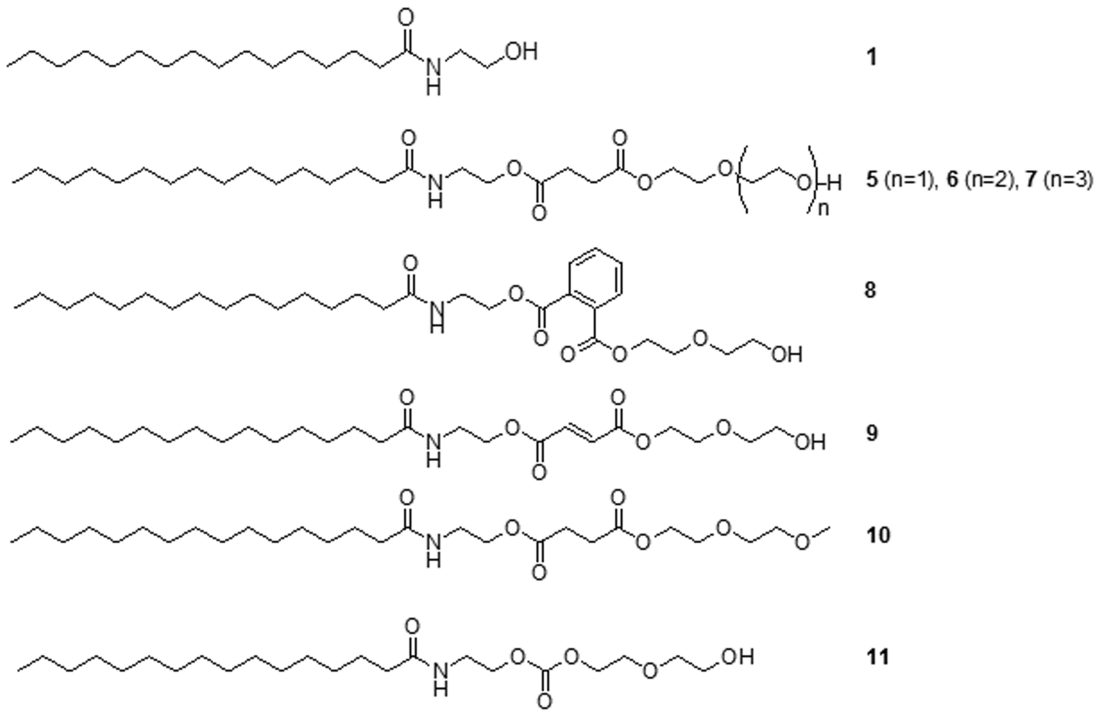

Figure 1. Structures of PEA (**1**) and its pegylated prodrugs diehtylene glycol-2-palmitamidoethyl succinate (**5**), triehtylene glycol-2-palmitamidoethyl succinate (**6**), tetraehtylene glycol-2-palmitamidoethyl succinate (**7**), diethylene glycol-2-palmitamidoethyl phtalate (**8**), diethylene glycol-2-palmitamidoethyl-(*E*)-fumarate (**9**), 2-(2-methoxyethoxy)ethyl-2-palmitamidoethyl succinate (**10**), and diethylene glycol-2-palmitamidoethyl carbonate (**11**).

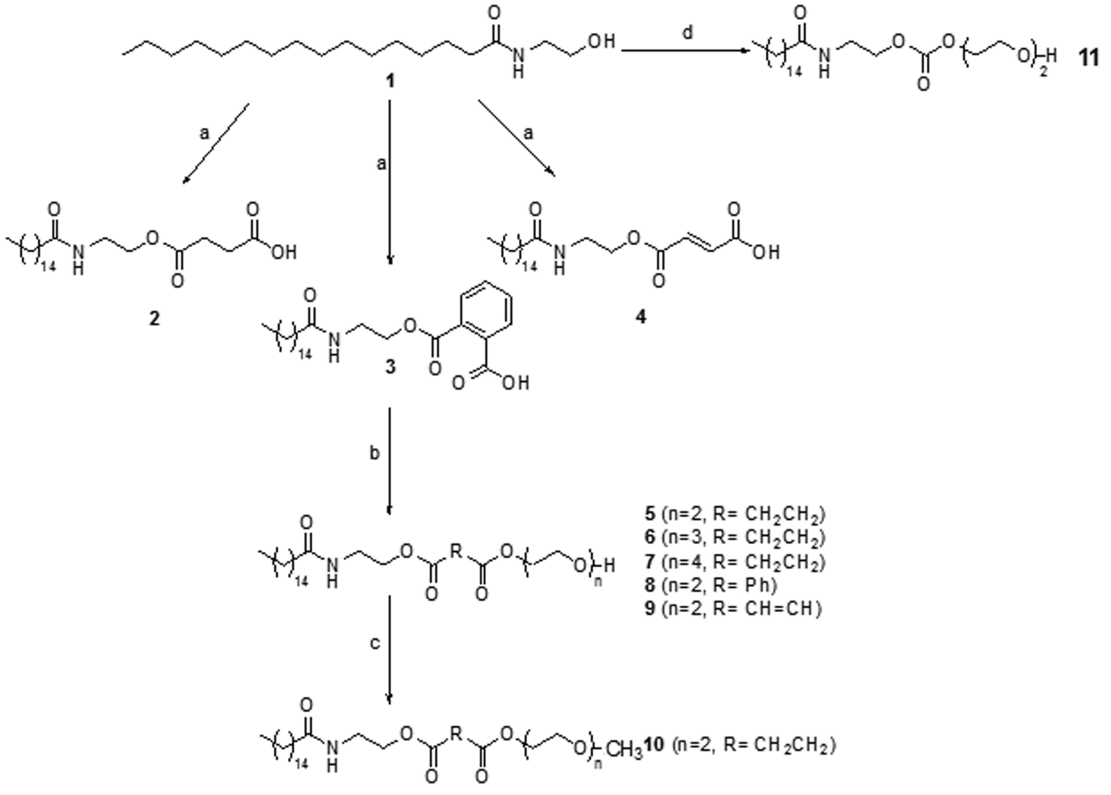

Figure 2. Synthesis of PEA derivatives. Reagent and conditions: (a) Succinic, phthalic, or maleic anhydride, THF, 50 °C, 3−5 h; (b) polyethylene glycol, DCC, DMAP, dry THF, room temperature, 4 h; (c) CH_3I, DMAP, dry acetone, 50 °C, 2 h; (d) CDI, diethylene glycol, dry dichloromethane, room temperature to 60 °C, 5 h.

Prodrug technologies are commonly employed to improve the membrane permeability or solubility of drugs, with wide application in skin delivery.[19] One of the most used strategies for prodrug design is the direct linkage to the parent drug of

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promoieties possessing an inherent permeation enhancing ability, in order to increase skin permeability and percutaneous absorption.[20] Among prodrugs, the use of a polyethylene glycol (PEG) promoiety is well-described, especially for skin delivery.[21,22] PEGs are capable of conferring an adequate aqueous stability as well as enhanced and targeted delivery to prodrugs. Moreover, the controlled enzymatic conversion into the parent drug and the amphiphilic properties make PEGs privileged promoieties in prodrug design.[21,23−26]

Starting from these lines of evidence and from the remarkable pharmacokinetic improvements obtained by previously investigated PEA prodrugs,[27,28] the aim of the present work was to synthesize a small library of PEA polyethylene glycol derivatives with sustained topical delivery and prolonged pharmacological efficacy (Figure 1). Skin accumulation was evaluated, *ex vivo*, by topical application of PEA and its prodrugs in mice. The detected PEA skin concentrations were rationalized by molecular modeling and *in vitro* chemical stability studies. Finally, PEA and its derivatives were tested *in vivo* for their anti-inflammatory and antihyperalgesic effects in a murine model of inflamed skin.

EXPERIMENTAL SECTION

General Procedures for the Synthesis of PEA Oligoethylene Derivatives. The general synthetic procedure adopted is described in Figure 2. Ultramicronized PEA was a kind gift by Epitech group srl (Saccolongo, PD, Italy). Succinic, phthalic, and maleic anhydrides, diethylene, triethylene, and tetraethylene glycols as well *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 1,1′-carbonyldiimidazole (CDI), and iodomethane were purchased by Sigma-Aldrich. Silica gel (0.04−0.063 mm, 70−230 mesh) was furnished by Macherey-Nagel (Duren, Germany). ^{1}H and ^{13}C NMR were recorded using a Mercury plus 400 MHz instrument (Varian Inc., Palo Alto, CA, USA). Trimethylsilane (TMS) was used as internal standard. Chemical shifts values are reported in δ units (ppm) relative to TMS (1%). The mass spectra were recorded using an API 2000 instrument (Applied Biosystem, Foster City, USA).

*4-Oxo-4-(2-palmitamidoethoxy)butanoic Acid (**2**), 2-[(2-palmitamidoethoxy)carbonyl]benzoic Acid (**3**), and (E)-4-Oxo-4-(2-palmitamidoethoxy)but-2-enoic Acid (**4**).* Intermediates **2**, **3**, and **4** were synthesized starting from 1.00 g of ultramicronized PEA (3.36 mmol), which was reacted with the appropriate anhydride (succinic, phthalic, or maleic anhydride, 4.36 mmol), in 25 mL of tetrahydrofuran (THF). The mixture was stirred at 50 °C for 3−5 h. Then, 10 mL of 2 N HCl were added, and the mixture was cooled to 0 °C. A precipitate was collected representing the desired intermediate. THF was then separated from the aqueous phase, which was extracted two more times with chloroform. The organic layers were collected, dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The crude product thus obtained was purified by chromatography using 9.5/0.5 chloroform/methanol as mobile phase and was combined with precipitate previously collected. The course of reaction and purification was monitored by glass TLC plates (0.25 mm 5 × 10 cm, Macherey-Nagel) after development with permanganate stain.

*4-Oxo-4-(2-palmitamidoethoxy)butanoic Acid (**2**).* Yield 83%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, 3H, J = 13.5 Hz), 1.22−1.40 (m, 24H), 1.60−1.65 (m, 2H), 2.20 (t, 2H, J = 15.1 Hz), 2.65 (t, 2H, J = 12.2 Hz), 2.71 (t, 2H, J = 12.2 Hz), 3.52−3.55 (m, 2H), 4.22 (t, 2H, J = 9.8 Hz), 6.00 (bs, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 14.68, 22.81, 25.83, 29.31, 29.36, 29.42, 29.50, 29.65, 29.72, 29.75, 29.76, 32.10, 36.01, 38.15, 63.50, 172.78, 173.18, 174.12. ESI-MS (m/z): 399.56.

*2-[(2-palmitamidoethoxy)carbonyl]benzoic Acid (**3**).* Yield 75%. ^{1}H NMR (400 MHz, CD_3OD): δ 0.81 (t, 3H, J = 12.8 Hz), 1.15−1.24 (m, 24H), 1.42−1.49 (m, 2H), 2.10 (t, 2H, J = 14.9 Hz), 3.45 (t, 2H, J = 9.6 Hz), 4.26 (t, 2H, J = 9.8 Hz), 7.51 (pt, 2H, J = 6.8 Hz), 7.58 (pd, 1H, J = 4.7 Hz), 7.7 (pd, 1H, J = 4.1 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 23.76, 26.95, 30.20, 30.45, 30.49, 30.60, 30.72, 30.78, 30.80, 33.09, 37.09, 39.30, 65.20, 129.67, 130.25, 132.06, 132.44, 133.30, 134.11, 169.80, 170.61, 176.66. ESI-MS (m/z): 448.30.

*(E)-4-Oxo-4-(2-palmitamidoethoxy)but-2-enoic Acid (**4**).* Yield 80%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90 (t, 3H, J = 13.2 Hz), 1.10−1.22 (m, 24H), 1.58−1.63 (m, 2H), 2.30 (t, 2H, J = 14.9 Hz), 3.60−3.65 (m, 2H), 4.35 (t, 2H, J = 10.0 Hz), 5.83 (bs, 1H), 6.30 (d, 1H, J = 11.7 Hz), 6.40 (d, 1H, J = 11.6 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 14.35, 22.91, 26.04, 29.45, 29.59, 29.74, 29.88, 29.93, 32.14, 32.62, 38.94, 64.47, 129.89, 131.87, 166.19, 166.81, 175.73. ESI-MS (m/z): 398.86.

*Diethylene Glycol-2-palmitamidoethyl succinates (**5**), Triehtylene Glycol-2-palmitamidoethyl Succinates (**6**), Tetraehtylene Glycol-2-palmitamidoethyl Succinates (**7**), Diethylene Glycol-2-palmitamidoethyl Phthalate (**8**), and Diethylene Glycol-2-palmitamidoethyl-(E)-fumarate (**9**).* Intermediates **2**−**4** (2.63 mmol) were reacted with the appropriate polyethylene glycol (diethylene glycol, triethylene glycol, and tetraethylene glycol, 3.95 mmol) in the presence of DMAP (0.03g, 0.26 mmol) and DCC (0.54 g, 2.63 mmol) in 30 mL of anhydrous tetrahydrofuran. The mixture was stirred at room temperature for 4 h. Then the precipitate formed was filtered off, and the filtrate was evaporated and reconstituted in 15 mL of chloroform. A saturated solution of $NaHCO_3$ was added, and the aqueous phase was extracted twice with chloroform. Organic phases were collected, dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The crude products were purified by chromatography using 9.5/0.5 chloroform/methanol as mobile phase.

*Diehtylene Glycol-2-palmitamidoethyl Succinate (**5**).* Yield 75%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, 3H, J = 13.3 Hz), 1.19−1.35 (m, 24H), 1.57−1.65 (m, 2H), 2.17 (t, 2H, J = 15.7 Hz), 2.62−2.70 (m, 4H), 3.50−3.54 (m, 2H), 3.61 (t, 2H, J = 4.4 Hz), 3.69−3.74 (m, 4H), 4.20 (t, 2H, J = 9.5 Hz), 4.27 (t, 2H, J = 8.4 Hz), 5.97 (bs, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 14.60, 23.90, 27.21, 29.97, 30.42, 30.61, 30.63, 30.80, 30.89, 30.92, 30.94, 33.23, 37.18, 39.51, 62.30, 64.35, 65.11, 70.86, 73.77, 174.08, 175.08, 176.76. ESI-MS (m/z): 487.67.

*Triehtylene Glycol-2-palmitamidoethyl Succinate (**6**).* Yield 70%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, 3H, J = 13.4 Hz), 1.24−1.38 (m, 24H), 1.58−1.64 (m, 2H), 2.18 (t, 2H, J = 15.0 Hz), 2.63−2.71 (m, 4H), 3.53−3.57 (m, 2H), 3.61 (t, 2H, J= 5.0 Hz), 3.66−3.74 (m, 8H), 4.21 (t, 2H, J = 9.8 Hz), 4.28 (t, 2H, J = 8.7 Hz), 6.00 (bs, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 15.95, 24.10, 26.13, 30.65, 30.71, 30.76, 30.84, 30.95, 31.03, 31.06, 31.08, 33.43, 37.40, 39.44, 62.35, 64.35, 64.84, 70.15, 71.54, 73.77, 174.08, 174.55, 176.76. ESI-MS (m/z): 531.72.

*Tetraehtylene Glycol-2-palmitamidoethyl Succinate (**7**).* Yield 65%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, 3H, J = 7.0), 1.26−1.31 (m, 24H), 1.58−1.64 (m, 2H), 2.18 (t, 2H, J = 15.4 Hz), 2.62−2.70 (m, 4H), 3.53−3.57 (m, 2H), 3.63 (t, 2H, J = 4.7) 3.65−3.75 (m, 12H), 4.22 (t, 2H, J = 9.7 Hz), 4.25 (t, 2H, J = 9.0 Hz), 6.0 (bs, 1H). ^{13}C NMR (100 MHz, CD_3OD):

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δ 15.97, 24.10, 26.15, 30.64, 30.71, 30.77, 30.85, 30.97, 31.02, 31.05, 31.09, 33.45, 37.41, 39.45, 62.40, 64.37, 64.84, 70.15, 71.54, 71.76, 73.77, 174.09, 174.57, 176.73. ESI-MS (m/z): 576.43.

*Diethylene Glycol-2-palmitamidoethyl Phtalate (**8**).* Yield 67%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.91 (t, 3H, J = 13.5 Hz), 1.22−1.36 (m, 24H), 1.60−1.65 (m, 2H), 2.22 (t, 2H, J = 15.4 Hz), 3.62−3.69 (m, 4H), 3.75−3.78 (m, 2H), 3.85 (t, 2H, J = 9.0 Hz), 4.47 (t, 2H, J = 10.2 Hz), 4.51 (t, 2H, J = 9.1 Hz), 6.3 (bs, 1H), 7,61 (pt, 2H, J = 7.2 Hz), 7.74 (pd,1H, J = 5.0 Hz), 7.82 (pd, 1H, J = 4.4 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 14.46, 23.75, 26.98, 30.24, 30.46, 30.49, 30.61, 30.73, 30.79, 30.80, 33.09, 37.09, 39.33, 62.20, 65.29, 66.10, 69.91, 73.70, 130.10, 132.47, 132.51, 133.19, 133.31, 168.97, 169.08, 176.64. ESI-MS (m/z): 536.73.

*Diethylene Glycol-2-palmitamidoethyl-(E)-fumarate (**9**).* Yield 62%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.91 (t, 3H, J = 13.5 Hz), 1.11−1.24 (m, 24H), 1.60−1.65 (m, 2H), 2.22 (t, 2H, J = 15.1 Hz), 3.49−3.53 (m, 2H), 3.61−3.66 (m, 4H), 3.8 (t, 2H, J = 5.2 Hz), 4.32 (t, 2H, J = 9.9 Hz), 4.41 (t, 2H, J = 8.5 Hz), 5.80 (bs, 1H), 6.31 (d, 1H, J = 11.7 Hz), 6.37 (d, 1H, J = 11.9 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 13.92, 22.08, 25.15, 28.60, 28.68, 28.76, 28.81, 28,87, 28.92, 29.03, 35.28, 37.26 63.06, 64.55, 65.26, 70.98, 73.67, 128.55, 131.57, 165.10, 166.45, 172.42. ESI-MS (m/z): 486.13.

*2-(2-Methoxyethoxy)ethyl-2-palmitamidoethyl Succinate (**10**).* To 0.260 g (0.547 mmol) of the intermediate **5** in 15 mL of dry acetone, DMAP (0.100 g, 0.820 mmol, 1.5 equiv) and iodomethane (0.05 mL, 0.820 mmol, 1.5 equiv) were added. The mixture was stirred at 50 °C for 2 h, then cooled to ambient temperature and concentrated *in vacuo*. The resulting solid was dissolved in chloroform, extracted with a saturated solution of $NaHCO_3$ and with 2 N HCl, dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. Crude product was purified by chromatography using ethyl acetate as mobile phase. Yield 71%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, 3H, J = 13.5 Hz), 1.18−1.35 (m, 24H), 1.55−1.64 (m, 2H), 2.18 (t, 2H, J = 15.5 Hz), 2.62−2.70 (m, 4H), 3.50 (s, 3H), 3.51−3.54 (m, 2H), 3.62 (t, 2H, J = 4.2 Hz), 3.71−3.75 (m, 4H), 4.22 (t, 2H, J = 9.6 Hz), 4.30 (t, 2H, J = 8.5 Hz), 6,00 (bs, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 14.62, 23.88, 27.24, 29.95, 30.50, 30.66, 30.68, 30.78, 30.91, 30.94, 30.98, 33.24, 37.19, 39.54, 58.91, 62.28, 64.32, 65.12, 70.84, 73.79, 174.01, 175.11, 176.79. ESI-MS (m/z): 502.82.

*Diethylene Glycol-2-palmitamidoethyl Carbonate (**11**).* To a solution of PEA (0.5 g, 1.167 mmol) in 17 mL of dry dichloromethane, CDI (0.3 g, 1.84 mmol, 1.1 equiv) was added portionwise. The reaction was maintained under stirring at room temperature for 2 h. Diethylene glycol (0.35 mL, 2.5 mmol, 1.5 equiv) was then added, and the reaction was warmed to 60 °C for 3 h. The solution was then cooled to room temperature and extracted with a saturated solution of $NaHCO_3$ and 2 N HCl, dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. Crude product was purified by chromatography using ethyl acetate as mobile phase. Yield 58%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, 3H, J = 13.4 Hz), 1.10−1.25 (m, 24H), 1.54−1.62 (m, 2H), 2.17 (t, 2H, J = 15.4 Hz), 3.55−3.58 (m, 2H), 3.62 (t, 2H, J = 8.4 Hz), 3.67−3.76 (m, 4H), 4.23 (t, 2H, J = 10.4 Hz), 4.33 (t, 2H, J = 9.1 Hz), 5.85 (bs, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 14.43, 23.74, 26.01, 26.99, 30.18, 30.32, 30.39, 30.47, 30.62, 30.73, 33.76, 30.78, 33.07, 34.97, 37.12, 42.91, 61.64, 62.19, 64.58, 70.13, 73.63, 163.36, 176.65. ESI-MS (m/z): 431.97.

Stability Studies. Prodrugs were dissolved in a solution of Tween 80 (5 mM)/methanol 10:1 (v:v), to a final concentration of 0.5 mg/mL. Aqueous phases were buffered at pH 5.0, mimicking the average pH of the skin, by acetate buffer. Solutions obtained were incubated at 37 °C. At predetermined intervals, aliquots of 100 μL were withdrawn, diluted 10 times with ethanol, evaporated *in vacuo*, and dried overnight on P_2O_5. PEA and its prodrugs were isolated by extraction and fractionation by silica gel chromatography as elsewhere described[29] and analyzed by HPLC as described below.

HPLC Analysis. HPLC analysis was performed on a Jasco apparatus (Jasco inc., Easton, MD, USA) equipped with a quaternary gradient module (PU 2089 Plus), a 25 μL rheodyne injection valve, and a multiwavelenght UV detector (MD 2010 Plus). The analytes were subjected to precolumn derivatization by dansyl chloride as previously described by Yagen and Burnstein.[30] Analytical parameters, determined in accordance with USP 38, are reported in Table 1. The specificity (absence of interfering peak from skin samples) was assessed as well.

Table 1. Analytical Parameter for Synthesized Derivatives

derivative	T_r (min)	linearity range (μg/mL)	R^2	resolution factor	% of recovery
PEA	28.5	0.32−10.00	0.988		95−115
5	25.4	0.45−10.00	0.981	2.06	88−99
6	23.2	0.50−8.00	0.981	3.25	85−98
7	22.6	0.50−8.00	0.976	3.60	85−94
8	27.4	0.47−9.30	0.984	1.05	89−99
9	24.9	0.51−8.70	0.987	2.16	90−97
11	24.6	0.49−9.10	0.972	2.30	84−94

Molecular Modeling. The conformational behavior of the PEA prodrugs was investigated by a clustered Monte Carlo procedure, which generated 1000 conformers by randomly rotating the rotors.[31] For each molecule, the lowest energy structure obtained was exploited in the following docking simulations. Docking simulations involved the previously generated hCES2 homology model[32] and were carried out using PLANTS,[33] which finds plausible poses through ant colony optimization algorithms (ACO). For all docking simulations, PLANTS was used with default settings and without geometric constraints. The search was focused on a 15.0 Å radius sphere around the catalytic Ser288 residue thus encompassing the entire enzymatic cavity. Poses were scored by the ChemPLP function using speed 1, and 10 poses were generated for each substrate. The best poses were chosen considering both the ChemPLP scores and the closeness between the substrate's labile function and Ser228. The selected complexes were then minimized keeping fixed all atoms outside a 15.0 Å radius sphere around the bound substrate, and the minimized structures were finally used to recalculate the PLANTS score functions.

Animals. Male Swiss mice weighing 25 to 30 g were purchased from Harlan (Udine, Italy). They were housed in stainless steel cages in a room kept at 22 ± 1 °C on a 12/12 h light/dark cycle. The animals were acclimated to their environment for 1 week, and they had *ad libitum* access to tap water and standard rodent chow. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M.

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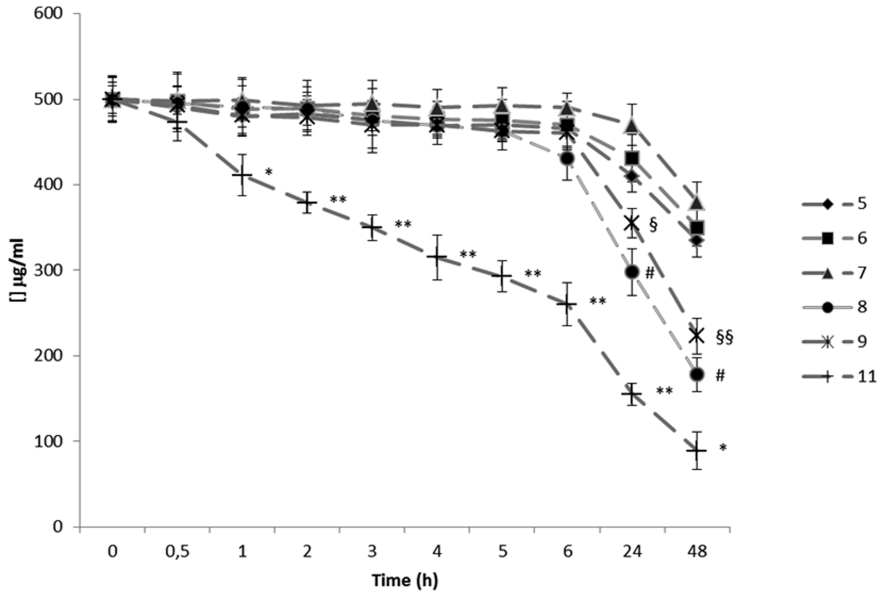

Figure 3. Chemical hydrolysis pattern for synthesized compounds. Pro-drug concentrations are expressed as micrograms of derivative per milliliter of solvent ± SEM (n = 3). Experiments were carried on at 37 °C and at pH 5.5. $^{§}p < 0.05$ concentration values significantly lower than **5**, **6** and **7**. $^{\#}p < 0.05$ concentration values significantly lower than **9**. $^{*}p < 0.05$, $^{**}p < 0.01$ concentration values significantly lower than **8**.

116192) as well as with European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986).

Skin Accumulation. Hydroalcoholic gels containing 0.5% (w/v) of PEA or equimolar amounts of prodrugs were used to assess skin permeation profiles. Gels were prepared dissolving Carbopol 980 (1% w/v, Lubrizol Corp., Wickliffe, USA) in 85 mL of distilled water at 90 °C under stirring. PEA and prodrugs were dissolved in 15 mL of absolute ethanol, and the resulting solutions were added, under magnetic stirring at room temperature, to the water phase until complete gelation. Drug delivery was pursued using Hill Top Chambers with webril pad (19 mm, Hill Top research, St. Petersburg, USA) loaded with 0.5 g of hydroalcoholic gel applied on the back of the shaved animal for 2 h (n = 5). At predetermined intervals (2, 4, 6, 24, 48, 72, and 96 h following patches applications), mice were euthanized by CO_2, and skin underlying permeation area was excised. Tissue samples were weighted and homogenized in a solution of methanol and a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF, 1 mM). Samples were centrifuged at 4 °C, for 30 min (1000 rpm). Crude products, obtained from the supernatant, were purified on silica gel and analyzed by HPLC as described above. The extraction method was validated in blank experiments by spiking freshly excised skin with a known amount of each analyzed compound. Recovery percentages are reported in Table 1. All the experiments were repeated in triplicate.

***In vivo* experiments.** Testing solutions were prepared by dissolving PEA (2 mg/mL) or equimolar doses of prodrugs (**5** = 3.25 mg/mL; **6** = 3.55 mg/mL; **7** = 3.84 mg/mL; **8** = 3.57 mg/mL; **9** = 3.24 mg/mL; **10** = 3.35 mg/mL; **11** = 2.88 mg/mL) in absolute ethanol; moreover, a mixture (**MIX**) composed of PEA (2 mg/mL) and equimolar concentrations of prodrugs **5** and **6** in absolute ethanol was also tested. Each solution (0.5 mL) was carefully applied by a Gilson pipet on mice paw. Control animal received only equal volumes of absolute ethanol. Paw edema was induced, 5 min later, by subplantar injection of 0.1 mL of sterile saline containing 1% λ-carrageenan into the hind paw. At predetermined intervals (2, 4, 6, 8, 24, 48, 72, and 96 h following injection), paw edema and mechanical hyperalgesia were evaluated (n = 6). Paw volumes were measured by a plethysmometer (Ugo Basile, Milan, Italy), and the increase in paw volume was assessed as the difference between the volume measured at each time point and the basal volume measured before carrageenan injection. In mechanical hyperalgesia we measured paw withdrawal threshold (g) by mechanical stimuli using the Randall-Selitto analgesimeter for mice (Ugo Basile, Italy). Latencies of paw withdrawal to a calibrated pressure were assessed on inflamed paw 1 day before ligation, and again 2−96 h following carrageenan injection; each paw was tested twice per session. Cut-off force was set at 100 g.

Statistical Analyses. Results are expressed as the mean ± SEM of n experiments. All analyses were conducted using Graph-Pad Prism (GraphPad Software Inc., San Diego, CA). The significance of differences between groups was determined by one way (for *ex vivo* and stability experiments) and two-way (for *in vivo* experiments) analyses of variance (ANOVA) followed by Bonferroni posthoc tests for multiple comparisons. Differences with $P < 0.05$ (*) were considered statistically significant.

RESULTS

Chemical Hydrolysis. Results obtained from stability studies are shown in Figure 3. Derivatives **5**, **6**, and **7** proved to be the most stable to chemical hydrolysis. No statistical differences were found in this group of derivatives. After 2 days, almost 20% of starting material was hydrolyzed. Noticeably, this loss did not cause the release of a comparable amount of PEA (data not shown). It can be concluded that chemical hydrolysis took place preferentially on the ester group distal from acylamide moiety, releasing intermediate **2** (Figure 2) rather than PEA. Chemical stability was strongly decreased in derivatives **8** and **9**. After 24 h in acidic media, 40% and 30% of starting material, respectively, was hydrolyzed. Percentage of hydrolysis increased at 48 h (65% for derivative **8** and 56% for derivative **9**). As previously evidenced, hydrolysis of prodrugs

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did not lead to comparable PEA release (data not shown). The least stable derivative to chemical hydrolysis was derivative **11**, rapidly decreasing initial concentration over time, with an overall hydrolysis rate of almost 80% after 48 h. Derivative **10** was not tested for chemical stability, being unsuitable for precolumn derivatization with dansyl chloride.

Molecular Modeling. As previously mentioned, docking simulations involved the hCES2 homology model since the hydrolytic capacity of the mammalian skin was found to be mostly ascribable to these isozymes.[34] Although hCES2 prefers esters with alkyl groups larger than acyl moieties, the simulated PEA esters should suitably fit the physicochemical properties of the enzymatic cavity since hCES2 better recognizes esters with very hydrophobic acyl groups and more polar alkyl moieties. As exemplified by the complex of **5** in Figure 4, all PEA prodrugs

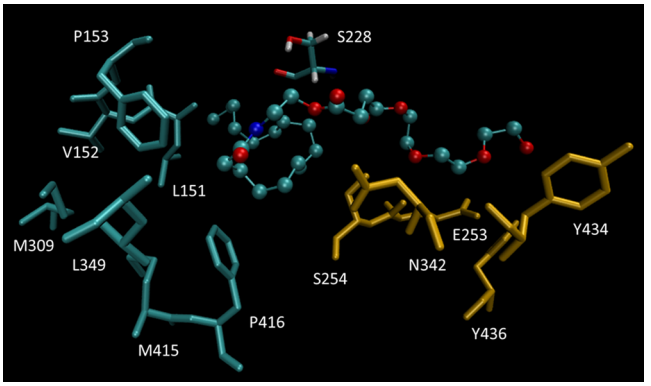

Figure 4. Main interactions stabilizing the putative complex between hCES2 and **5**. The residues interacting with the alkyl chain are displayed in gold, while those contacting the acyl chain are in cyan.

assume a similar pose within the hCES2 cavity since the palmitoyl chain is inserted into a hydrophobic subpocket where it contacts a vast set of apolar residues (e.g., Leu151, Val152, Phe153, Phe184, Met309, Leu349, Met415, and Phe416), while the more polar alkyl moiety is accommodated within a subpocket where the PEG units can stabilize H-bonds with several polar surrounding residues (e.g., Glu253, Ser254, Asn342, Tyr434, Tyr436, and Tyr490).

In all computed complexes, the catalytic Ser228 residue conveniently approaches the carbonyl carbon atom of the ester groups closest to the amide function thus indicating that the hCES2 hydrolysis directly liberates PEA. While the apolar contacts stabilized by the acyl chain are constantly observed in all computed complexes, marked differences are seen in the polar interactions elicited by the alkyl moieties, the number of elicited H-bonds being roughly proportional to the observed hydrolysis. In detail, the prodrugs, which are efficiently hydrolyzed (see Figure 7) elicit clear H-bonds with both Glu253 and Ser254, which may constrain the substrate in a pose stably conducive to the catalysis. In contrast, the prodrugs hydrolyzed with difficulty stabilize only weaker interactions with Tyr434 or Tyr436.

Although the low number of investigated prodrugs prevents the development of statistically robust relationships, a simple correlative study was carried out with a view to revealing those factors significantly influencing the PEA skin release. The study was based on the PEA concentrations at 6 h because they appear to be mostly due to the enzymatic hydrolysis as well as to the prodrug skin permeation, while the concentrations after 24 and 48 h are vastly affected by chemical hydrolysis (see above). Compound **11** was not included in the analysis since it was found to be chemically unstable even at 6 h. In detail, Table 3 reports some relevant docking score and physicochemical descriptors and reveals (1) the correlation between the considered PEA concentrations and the computed docking ChemPlp scores, thus confirming that the PEA skin release can be mostly ascribed to the hCES2 catalyzed hydrolysis; (2) the inverse correlation between PEA concentrations and molecular weights, which is understandable in terms of skin permeation according to the well-known Potts and Guy model;[35] and (3) the correlation between PEA concentrations and lipophilicity (as computed by the MLP approach), which can be easily explained in terms of both skin permeation and enzymatic recognition. Notably, the same descriptors afford dramatically worse relationships with the PEA concentrations at 24 or 48 h thus confirming that only those at 6 h are amenable to reliable QSAR analyses.

Skin Accumulation. Time-course skin accumulation values for PEA and its prodrugs are depicted in Figure 5. It has to be

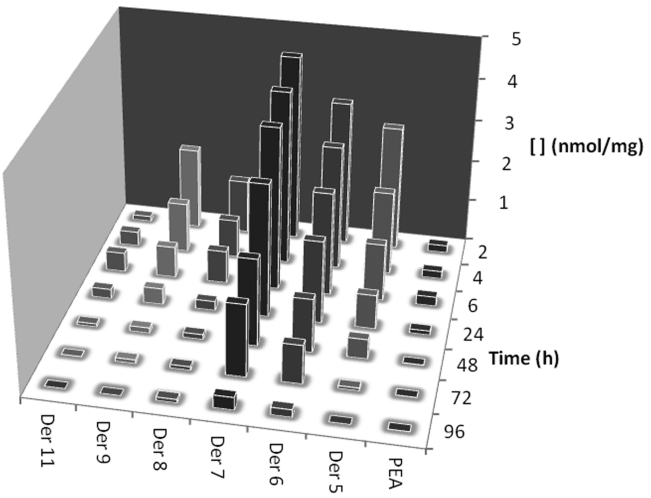

Figure 5. Compared accumulation profiles between PEA and synthesized prodrugs. Amount accumulated is reported as average nanomoles of PEA per milligram of excised skin (n = 5).

noticed that PEA skin concentrations were constant during the first 6 h after topical applications, were markedly reduced at 24 h, and, thereafter, only traces of PEA were detected. A different accumulation pattern was highlighted for synthesized prodrugs. As evidenced in Figure 5 most of the prodrugs accumulated to a major extent (10- to 15-fold) when compared to PEA, and some of them (derivative **6** and **7**, in particular) were detected in relatively high amount also 5 days after removal of patches. Only derivative **11** showed a skin accumulation pattern comparable to the parent drug. Cutaneous accumulation for derivative **10** was undeterminable since the molecule is unsuitable for precolumn derivatization.

The amount of PEA released from each synthesized prodrug was assessed in the same time interval. Results obtained are shown in Table 3. Synthesized prodrugs can be roughly divided into three different groups.

Compounds **7**, **8** and **11** released a really low amount of PEA during the entire time-course. PEA skin accumulation determined by prodrug **9** was significantly higher than the parent drug only 6 h after topical administration. However, a

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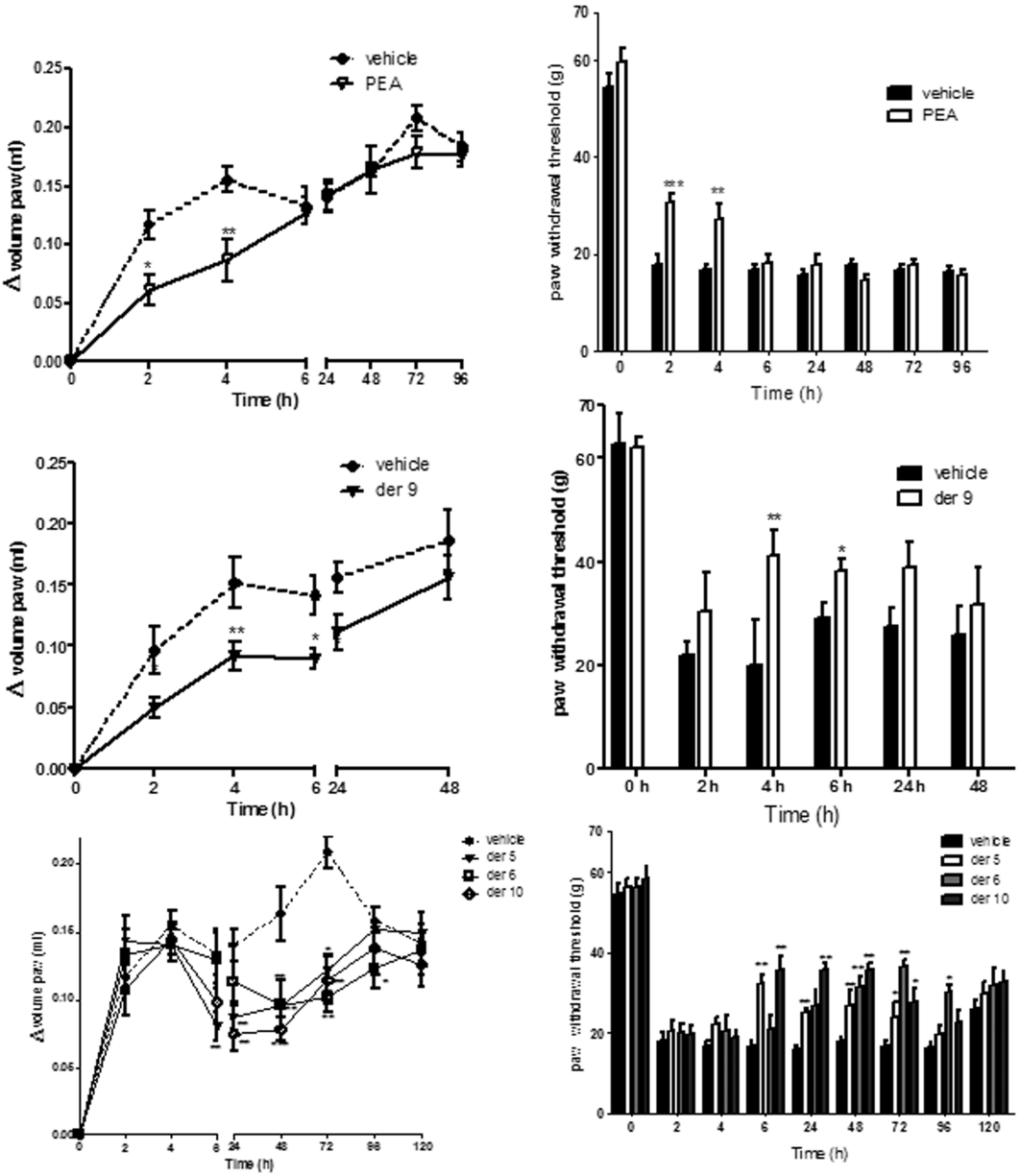

Figure 6. Effect of local application of vehicle (CTR), PEA, and prodrugs **5**, **6**, **9**, and **10** on paw edema (A,C,E) and mechanical hyperalgesia (B,D,F). Data represents mean ± SEM of six mice. *p < 0.05, **p < 0.01, and ***p < 0.001 vs CTR.

remarkable skin accumulation of PEA was obtained from derivatives **5**, **6**, and **10**. Inside this group, prodrugs **5** and **10** showed comparable release profiles, while prodrug **6** determined a prolonged PEA release with higher accumulation rates 48 and 96 h following application (p < 0.05 vs prodrugs **5** and **10**)

Pharmacological Studies. As expected, carrageenan injection into the mice paw produced both significant hyperalgesia as determined by a reduction in withdrawal latencies (s) following mechanical stimulation (Figure 6B,D,F, black bars) and evident paw edema as determined by an increase in paw volume (Figure 6A,C,E, black circles). PEA treatment (1 mg/paw) markedly reduced mechanical hyperalgesia and paw edema in a time-dependent manner, as shown by the increase in paw withdrawal latency and by the reduction of paw volume (Figure 6A,B). In particular, antihyperalgesic and anti-inflammatory effects were observed at 2 and 4 h following application, whereas no effect was later detectable. In accordance with skin accumulation experiments, derivatives **7**, **8**, and **11** did not demonstrate any pharmacological effect (data not shown). Derivative **9** (Figure 6C,D) showed moderate and short-term effects. Hyperalgesia and paw volume were both reduced 4 h after applications, but pharmacological activity fell during 24 h. In contrast, derivatives **5**, **6**, and **10** (Figure 6E,F) demonstrated delayed but prolonged antihyperalgesic and anti-inflammatory effects. In particular, derivatives **5** and **10** showed significant antihyperalgesic and anti-inflammatory effects between 6 and 24 h following topical application. Derivative **6** further delayed the pharmacological effect to 48 h after application, maintaining efficacy until day 5. Finally, no swelling effect on the paw was observed when the topical application of prodrugs was not followed by carrageenan injection (negative control, data not shown). Aiming to obtain a formulation characterized by early and prolonged efficacy, we tested a mixture (**MIX**) composed of PEA, **5**, and **6**, in the same experimental conditions. As expected, the mixture showed a

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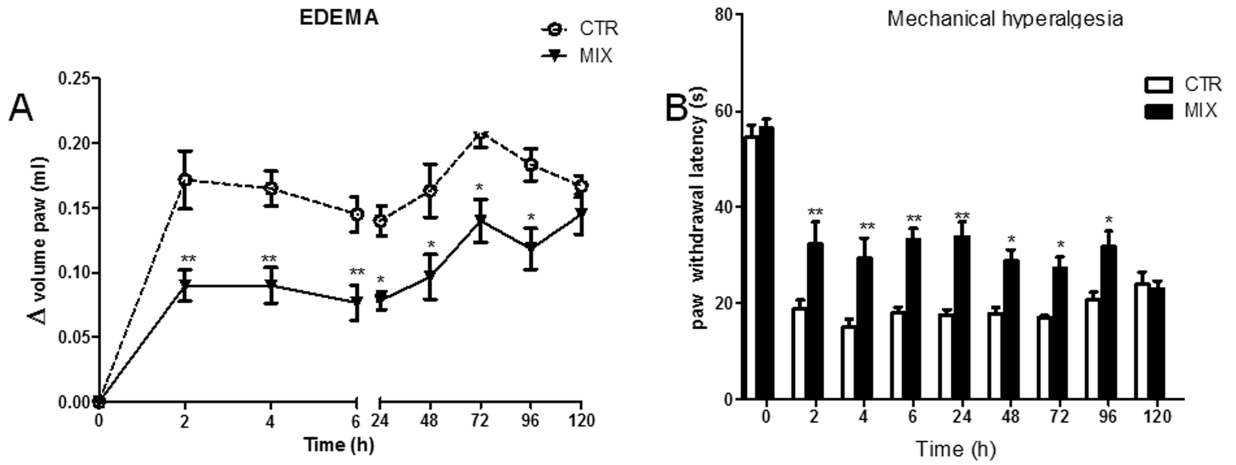

Figure 7. Effect of local application of vehicle (CTR) and PEA + prodrug **5** + prodrug **6** (**MIX**) on mechanical paw edema (A) and mechanical hyperalgesia (B). Data represents mean ± SEM of six mice. *p < 0.05, **p < 0.01 and vs CTR. See experimental section for methods used.

rapid onset of action along with prolonged efficacy (Figure 7), thus dramatically improving the pharmacological profile of the parent drug.

DISCUSSION

PEA, an endogenous PPAR-α agonist, is anticipated to become, like the adelmidrol (azealic acid diethanolamide), a key tool of efficacious fatty acid derivatives for topical treatment of inflamed skin.[15−17] Recently, several studies have pointed out that PPAR-α agonist actively reduces skin lesion,[12,14,36] and some of them have already been delivered to the clinical scenario, both for human or veterinary use.[16−18,37] PEA is one of the most attractive PPAR-α agonists, representing a naturally occurring anti-inflammatory molecule with wide ranging applications in prevention and treatment of skin pathologies characterized by lack of local and systemic adverse side effects, especially in comparison with standard steroidal anti-inflammatory therapies.[12,16,17,36] Despite these favorable properties, the use of topically administered PEA is strongly limited by its disadvantageous pharmacokinetic properties, leading to short-term effects, as also evidenced in the present work. Both anti-inflammatory and antihyperalgesic effects of PEA rapidly disappear after topical application in the murine model of skin inflammation herein used (Figure 6A,B). This short-term effect can be justified by PEA inactivation by two intracellular lipid amidases: *N*-acylethanolamine acid amidase (NAAA)[38] and fatty acid amide hydrolase (FAAH).[39] Recently, Sasso et al.[40] showed that a potent and selective NAAA inhibitor protects endogenous FAEs from NAAA-catalyzed degradation in inflamed tissues following local administration. Moreover, simultaneous topical administration of PEA and FAAH inhibitors is capable of boosting the effects of the fatty acid ethanolamide.[41]

Hence, the objective of the present work was to synthesize pegylated PEA prodrugs for a sustained topical accumulation and delivery as well as prolonged activity. Synthesized prodrugs showed, for the most part, an increased accumulation in skin if compared with the parent molecule but were discriminated by their ability to release PEA and exert pharmacological properties. The succinate spacer proved to be the most suitable. In fact, derivative **5** accumulates to a major extent if compared with its homologues (**8**, **9**, and **11**). These results cannot be solely rationalized by molecular weight and partition coefficient values, which are very similar among library members (Table 2). It can be postulated, for derivatives **8** and **9**, that molecular constraint, introduced by a malonate or phthalate linker negatively influence skin diffusion, thus limiting tissue accumulation. Molecular rigidity has been previously described as a penalizing factor for skin permeation.[42,43] However, while a carbonate linker has been widely described and profitably used in prodrug design, derivative **11** showed an unfavorable accumulation profile (Figure 5) and lacked pharmacological effects. This prodrug proved to be very susceptible to chemical hydrolysis (Figure 3), probably leading to a fast and uncontrolled release of PEA, prior to reaching suitable concentrations in the viable epidermis. As reported by Vlieghe et al.,[44] carbonates are susceptible to molecular rearrangements in the presence of a hydroxyalkyl side chain.

Table 2. Correlation between PEA Skin Accumulation at 6 h and Some Relevant Descriptors Including Docking Scores

derivative	[PEA] 6 h	ChemPlp (kcal/mol)	M.W. (g/mol)	logP_MLP
5	0.274	−92.64	487.67	6.18
6	0.073	−89.75	531.72	5.57
7	0.093	−68.75	575.78	5.99
8	0.105	−75.58	535.71	6.16
9	0.301	−101.37	485.65	6.13
10	0.324	−97.23	501.67	6.94
correlation (r)		0.79	−0.84	0.73

As expected, the size of the polyethylene glycol promoieties influences the pharmacokinetic profile and pharmacological activity of synthesized prodrugs.[21] An increase in the number of ethylene glycol units leads to improved skin accumulation (Figure 5). Tissue concentrations found for prodrug **7** are significantly higher in comparison with derivatives **6** and **5**. The increase in molecular weight is compensated for by a more favorable partition coefficient. Despite this suitable accumulation profile, derivative **7** is totally inactive, while its lower homologues showed a substantial pharmacological activity with delayed and prolonged anti-inflammatory and antihyperalgesic effects (Figure 6C−F). These results can be rationalized by molecular modeling results, which highlighted how the catalytic Ser228 residue of the esterase approaches the carbonyl carbon atom of the ester groups closest to the amide function (Figure

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Table 3. Comparison between PEA Skin Accumulation and Release from Synthesized Prodrugs[a]

	time course						
compound	[ ] 2 h	[ ] 4 h	[ ] 6 h	[ ] 24 h	[ ] 48 h	[ ] 72 h	[ ] 96 h
PEA	0.169 ± 0.025	0.173 ± 0.022	0.218 ± 0.041	0.093 ± 0.011	nd	nd	nd
PEA from **5**	0.071 ± 0.011	0.111 ± 0.016$^{\#}$	0.274 ± 0.034*	0.212 ± 0.050**	0.174 ± 0.036***	0.139 ± 0.066***	0.051 ± 0.023*
PEA from **6**	0.048 ± 0.027$^{\#}$	0.076 ± 0.012$^{\#}$	0.073 ± 0.054	0.312 ± 0.077**	0.343 ± 0.046***	0.268 ± 0.046***	0.141 ± 0.036**
PEA from **7**	nd	nd	0.093 ± 0.044$^{\#}$	0.083 ± 0.060	0.034 ± 0.006*	0.028 ± 0.005*	0.023 ± 0.016
PEA from **8**	0.012 ± 0.009$^{\#}$	0.054 ± 0.014$^{\#}$	0.105 ± 0.025$^{\#}$	0.088 ± 0.030	0.027 ± 0.016	nd	nd
PEA from **9**	0.115 ± 0.041$^{\#}$	0.235 ± 0.029	0.301 ± 0.047*	0.106 ± 0.031	0.009 ± 0.003*	nd	nd
PEA from **10**	0.062 ± 0.029$^{\#}$	0.120 ± 0.012$^{\#}$	0.324 ± 0.043*	0.245 ± 0.067**	0.216 ± 0.045***	0.147 ± 0.042***	0.062 ± 0.025*
PEA from **11**	0.018 ± 0.010$^{\#}$	0.041 ± 0.004$^{\#}$	0.074 ± 0.006$^{\#}$	0.033 ± 0.003$^{\#}$	0.014 ± 0.007	nd	nd

[a]Time course concentration is expressed in nanomoles of PEA per milligram of tissue ± SEM (n = 5) at different times. When outside the linearity range of the analytical method used, concentrations were considered not determinable (nd). *p < 0.05, **p < 0.01, and ***p < 0.001 accumulation values significantly higher than PEA. $^{\#}p$ < 0.05 accumulation values significantly lower than PEA.

4). Thus, an increase in side chain size is responsible for a slower hydrolysis of the ester bond (Table 2). For these reasons, despite the higher skin concentrations reached, derivative **7** is unable to release biologically active amounts of PEA (Table 3). Contrariwise, derivative **5** and **6** act as depot prodrugs for sustained release, prolonging the therapeutic efficacy of PEA after topical administration. In fact, a mixture of compounds **5** and **6** and PEA, at equimolar dose, resulted in a fast outbreaking and long-lasting anti-inflammatory topical formulation, as depicted in Figure 7. Finally, the evidence shows that derivative **10** maintains the same advantageous pharmacological profile as prodrug **5**. Taken together our data showed (i) that prodrugs have an efficacy comparable to local or systemic administration of standard anti-inflammatory drugs (i.e., glucocorticoids);[45,46] and (ii) that local pretreatment with PEA and its prodrugs maintain cellular homeostasis by acting as a mediator of resolution of inflammatory processes and by modulating the behavior of mast cells and microglia.[47]

CONCLUSIONS

In this work, we investigated a prodrug approach for topical delivery of PEA as anti-inflammatory drug. The chemical combination of the parent drug with specific polyethylene glycol moieties, using the proper spacer, proved to be a viable approach to increase skin accumulation and overcome pharmacokinetic limitations to the use of topical PEA. Therefore, a combination of the parent drug and the most active prodrugs led to a retarding topical formulation, characterized by rapid onset of action and long duration. Collectively these results suggest the use of polyethylene glycol prodrugs of PEA as suitable candidates for the treatment of skin inflammatory diseases. Moreover, considering the role of lipids as prodrug carriers,[48] and the well-described use of lipid−PEG conjugates for the design drug delivery systems,[49] results obtained pave the way for the use of PEA and its derivatives as components of codrugs and particles for sustained and/or targeted delivery.

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Author Contributions

[||]These authors contributed equally to this work. Study conception and design: D.T., R.R., C.O., G.V., and A.C. Synthesis of prodrugs: D.T. and C.O. Acquisition of data: R.R., C.D.C., C.A., and G.L.R. (*in vivo* experiments and skin accumulation experiments); D.T. and C.O. (skin accumulation experiments and chemical stability); A.S. (NMR and mass spectrometry); S.L. (HPLC analysis); A.M. and G.V. (computational data). Drafting of manuscript: D.T., R.R., C.O., G.V., and A.C. Critical revision: A.C. and F.D.V. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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