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Multiple drug-delivery strategies to enhance the pharmacological and toxicological properties of Mefenamic acid

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ABSTRACT

Objective: To improve the biological and toxicological properties of Mefenamic acid (MA), the galactosylated prodrug of MA named MefeGAL was included in polymeric solid dispersions (PSs) composed of poly(glycerol adipate) (PGA) and Pluronic® F68 (MefeGAL-PS). MefeGAL-PS was compared with polymeric solid formulations of MA (MA-PS) or a mixture of equal ratio of MefeGAL/MA (Mix-PS).
Methods: The *in vitro* and *in vivo* pharmacological and toxicological profiles of PSs have been investigated. In detail, we evaluated the anti-inflammatory (carrageenan-induced paw edema test), analgesic (acetic acid-induced writhing test) and ulcerogenic activity in mice after oral treatment. Additionally, the antiproliferative activity of PSs was assessed on *in vitro* models of colorectal and non-small cell lung cancer.
Results: When the PSs were resuspended in water, MefeGAL's, MA's and their mixture's apparent solubilities improved due to the interaction with the polymeric formulation. By comparing the *in-vivo* biological performance of MefeGAL-PS with that of MA, MefeGAL and MA-PS, it was seen that MefeGAL-PS exhibited the same sustained and delayed analgesic and anti-inflammatory profile as MefeGAL but did not cause gastrointestinal irritation. The pharmacological effect of Mix-PS was present from the first hours after administration, lasting about 44 hours with only slight gastric mucosa irritation. *In-vitro* evaluation indicated that Mix-PS had statistically significant higher cytotoxicity than MA-PS and MefeGAL-PS.
Conclusions: These preliminary data are promising evidence that the galactosylated prodrug approach in tandem with a polymer-drug solid dispersion formulation strategy could represent a new drug delivery route to improve the solubility and biological activity of NSAIDs.

1. Introduction

Prodrugs are bioreversible derivatives of drug molecules with little or no pharmacological activity that are converted *in vivo* into therapeutically active compounds by enzymatic pathways and/or chemical transformations [1]. The prodrug approach is used as a valid strategy to optimize physicochemical, biopharmaceutical and/or pharmacokinetic characteristics of those parent drugs which suffer from issues such as stability, toxicity, solubility, permeability, and drug targeting [2]. Since the primary structure of the parent drug is not altered, the synthesis of prodrugs is less difficult than that of its analogues. Approximately 10–14 % of drugs approved worldwide can be classified as prodrugs [2]. The

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properties of the resulting derivatives can be accurately tailored through structural modification using a promoiety [3,4].

Considering the broad class of nonsteroidal anti-inflammatory drugs (NSAIDs), it has been found that D-galactose is promising promoiety [5–7].

The galactosylated prodrug approach can be seen as a simple and powerful problem-solving technique adoptable to overcome the drawbacks of NSAIDs. In this regard, our group has shown that the chemical conjugation of NSAIDs to D-galactose satisfied a series of requirements in terms of physicochemical features. None of these conditions would be met by free NSAIDs [5].

By employing D-galactose as a carrier for ibuprofen, ketoprofen, flurbiprofen, indomethacin [5], paracetamol [8], aceclofenac [9], the respective galactosylated prodrugs exhibited a prolonged and time-delayed pharmacological activity compared with their parent drugs. The development of NSAIDs with an extended profile could be particularly advantageous in chronic inflammatory diseases: a single administration of the galactosylated derivative could replace the repeated use of NSAIDs, thereby reducing side effects or eliminating the co-prescription of other drugs (e.g., proton pump inhibitors, PPIs) [10–13].

The valuable role of D-galactose in the toxicological profile of NSAIDs has also been proven the presence of the sugar moiety reduced gastrointestinal injuries, both by preventing the ion-trapping phenomenon and by decreasing the blockade of prostaglandin biosynthesis and mucus production in the stomach. Therefore, the design of galactosylated prodrugs proved to be a fruitful approach for NSAIDs, confirming the importance of drug delivery systems in experimental pharmaceutics and clinical medicine [5,6].

Polymeric solid dispersions are simple mixtures of poorly water-soluble active ingredients, or more generically of a drug, and a hydrophilic polymer [14,15].

By minimizing drug recrystallization (through polymer-drug interactions), the action of the polymer is to induce and stabilize the amorphous state of the drug in the solid state. Consequently, the effect of polymeric solid dispersions is to enhance drug water dissolution and solubility [16–20].

In addition, it has been demonstrated that the use of a surfactant in tandem with a polymer in a solid dispersion could improve drug-polymer miscibility, reduce drug recrystallization tendency, but also it may improve solid dispersion wettability, subsequently, increasing water dissolution of the drug [21,22].

Recently, multi-drug co-administration systems have emerged as a promising approach to combat cancer, as synergistic effects and reduced side effects are expected; however, their use is still at a rather pioneering stage [23]. While the application of formulation strategies, such as the prodrug strategy-plus-particle system, has not been yet adopted.

In the present work, for the first time, we have combined the advantages of the galactosylated prodrug approach in tandem with a polymeric solid dispersion (PS) formulation strategy.

Mefenamic acid [2-[2,3-dimethylphenyl)amino]benzoic acid (MA) is a common NSAID belonging to the class of anthranilic acid derivatives used for the relief of postoperative and traumatic inflammation and swelling, antiphlogistic and analgesic treatment of rheumatoid arthritis, and antipyretic in acute respiratory tract infection [24].

NSAIDs including MA are reported to have antiproliferative activity in several *in vitro* cancer models, including in breast [25,26], prostate [27], stomach [28,29], liver [30], lung [31], and colon cancer cell lines [32,33]. The cytotoxicity of NSAIDs has primarily been associated with the induction of apoptosis and believed to be induced via several potential mechanisms, including COX inhibition, inhibition of prostaglandin H synthase, reduction in epidermal growth factor (EGF) and the upregulation of tumour suppressor genes; p53, MAP kinase phosphatase-3 and PTEN [30–34]. Moreover, *in vivo* evidence focused on colorectal models has demonstrated that MA, together with many other NSAIDs, was able to reduce the formation of both colon adenomatous Polyps and cancers in experimental animals; in other *in vivo* models they inhibited the growth and clinical expression of transplanted tumours and metastatic spread, and potentiated the antitumor effects of immunotherapy, radiotherapy, and cytotoxic drug therapy [35,36]. Although MA is available in the market under different forms, both tablets and suspensions, its oral administration can cause serious gastric side effects which can lead up to gastrointestinal bleeding [37].

To overcome these known drawbacks and improve the biological properties of MA, we reported the preparation and characterization of a galactosylated prodrug of MA prepared by simple polymeric solid resuspension. Specifically, the galactosylated prodrug of MA named MefeGAL (synthesis reported in the previous literature) [38] was included in polymeric solid dispersions (PSs) composed of poly(glycerol adipate) (PGA) and Pluronic® F_{68} (MefeGAL-PS). PGA is an amphiphilic, biodegradable and functionalizable polymer previously used as carrier in amorphous solid dispersions of MA [39,40]. While Pluronic® F_{68} is water soluble, commercial non-ionic polymeric surfactant extensively used to improve drugs solubility and in solid dispersions [41–46]. MefeGAL-PS was compared with polymeric solid formulations of MA (MA-PS) or a mixture of equal ratio of MefeGAL/MA (Mix-PS). Moreover, the *in vitro* and *in vivo* pharmacological and toxicological profiles of PSs have been investigated. In detail, we evaluated the anti-inflammatory (carrageenan-induced paw edema test), analgesic (acetic acid-induced writhing test) and ulcerogenic activity in mice after oral treatment. Additionally, the antiproliferative activity of PSs was assessed on *in vitro* models of colorectal and non-small cell lung cancer.

2. Materials and methods

2.1. Materials

All chemical reagents were used as obtained, without further purification, unless otherwise stated and purchased from Sigma-Aldrich. All solvents were purified and degassed before use. THF dry was freshly distilled from sodium/benzophenone. MefeGAL was synthesized as previously reported [38]. The plasticware for cultures was obtained from Falcon (Becton Dickinson, Franklin Lakes, NJ, USA).

2.2. Preparation of PS

MA-PS. MA (2.5 mg), PGA (33 mg for *in vivo* studies/11 mg for *in vitro* studies), and Pluronic F_{68} (33 mg for *in vivo* studies/11 mg for *in vitro* studies) were weighed into vials and dissolved in dry THF (2 mL). THF was evaporated in a fumehood overnight. The obtained MA film was dispersed in 2.5 mL of warm water (35°C), vortexed (2 minutes) then sonicated for 2 minutes and vortexed again for 2 minutes. The final concentration of loaded MA, measured with a UV-Vis spectrophotometer as described below, was 1.13 mg/mL.

MefeGAL-PS. MefeGAL (2.5 mg), PGA (33 mg for *in vivo* studies/11 mg for *in vitro* studies), and Pluronic F_{68} (33 mg for *in vivo* studies/11 mg for *in vitro* studies) were weighed into vials and dissolved in dry THF (2 mL). THF was evaporated in a fumehood overnight. The resulting MefeGAL film was dispersed in 1.515 mL of warm water (35°C), vortexed (2 minutes) then sonicated for 2 minutes and vortexed again for 2 minutes. The final concentration of loaded MefeGAL, measured with a UV-Vis spectrophotometer as described below, was 2.45 mg/mL.

Mix-PS. MA (1.25 mg), MefeGAL (1.25 mg), PGA (33 mg for *in vivo* studies/11 mg for *in vitro* studies), and Pluronic F_{68} (33 mg for *in vivo* studies/11 mg for *in vitro* studies) were weighed into vials and dissolved in dry THF (2 mL). THF was evaporated in a fumehood overnight. The Mefenamic/MefeGAL film was dispersed in 2.5 mL of warm water (35°C), vortexed (2 minutes) then sonicated for 2 minutes and vortexed again for 2 minutes. The estimated final concentration of loaded MA and MefeGAL, measured with a UV-Vis spectrophotometer as described below, was around 1.66 and 2.26 mg/mL, respectively.

For drug water solubility enhancement study, the MA or MefeGAL or

Mix film was dispersed in water (6.25 mL), for a final concentration of 400 μg/mL of drug, shaken then sonicated for 20 minutes. Samples have been analysed for ΔA % calculation (see below) and used *in vitro* tests after filtration.

2.3. Apparent drug water solubility improvement: ΔA % calculation

The absorbance of the drug/prodrug-PSs resuspended in water was measured using a UV–Vis spectrophotometer multi-well plate reader (Epoch 2 Microplate Spectrophotometer, Biotek) at $\lambda_{max} = 292$ nm (MA) or 354 nm (MefeGAL). The apparent solubility (ΔA %) of each formulation was determined using equation (1), previously developed by Sanna *et al.* and Jacob et al. [47–49].

$$\Delta A\% = \frac{\Delta A}{A_0} \times 100 = \frac{(A - A_0)}{A_0} \times 100$$

(A_0) Absorbance of the drugs alone in water.

(A) Absorbance of the aqueous solutions of drug/prodrug-PSs (after subtraction of polymers absorbance in water at the two wavelengths, respectively).

2.4. Drug loading

The drug-loaded polymeric solid dispersion (MA-PS, MefeGAL-PS, and Mix-PS), resuspended in water, were appropriately diluted (20, 15, and 30 mL of methanol, respectively) and analyzed with V-750 UV-Vis spectrophotometer (Jasco) in order to measure the exact amount of encapsulated drug/prodrug. The concentration of MA and/or MefeGAL expressed as mg/mL, was determined by quantifying the absorption of the diluted solution at the wavelength of 292 nm for MA and 354 nm for MefeGAL and using the corresponding calibration curves obtained with standard solutions. Seven-point calibration standards (0.025, 0.050, 0.080, 0.100, 0.125, 0.250, 0.500 mM) were prepared by dilution from 10 mM stock solutions (in methanol) of MA and MefeGAL ($r^2 > 0.99$).

2.5. Dynamic light scattering (DLS)

Dynamic light scattering was used to determine nanoaggregates size produced by PS formulation resuspension in water using a Zetasizer Nano spectrometer (Malvern Instruments Ltd.) equipped with a 633 nm laser at a fixed angle of 173° and a Wyatt DyanPro DLS Plate Reader. Samples were equilibrated for 30 s at 25 °C prior to measurement.

2.6. Animal model

Ten-week-old male Swiss CD1 mice weighing 30–35 g were acquired from Charles Rivers (Calco, Italy). They were maintained for 1 week under controlled environmental conditions (22 ± 1 °C, 12/12 h light/dark cycle), with *ad libitum* access to water and a standard rodent chow diet. All procedures involving the mice were conducted in conformity with Institutional Guidelines and are in line with the Italian Ministry of Health and the relevant guidelines of the European Communities Council Directive. The procedures reported here were approved by the Institutional Committee on the Ethics of Animal experiments (CSV) at the University of Naples "Federico II" and by the Ministry of Health under protocol no. 0084607. At the end of tests, the animals were euthanized by CO_2 inhalation overdose.

2.6.1. Experimental groups and procedures

Mice were divided into six groups of six animals each. All drugs are dissolved in aqueous solution of sodium carboxymethyl cellulose (CMC, 0.5 % w/v) used as a vehicle. Animals received vehicle or drug treatments orally. The groups are indicated as follow:

- Group I: served as control group (CTRL), receiving the vehicle (CMC).
- Group II: receiving MA at a dose of 10 mg/kg.
- Group III: receiving MefeGAL at a dose of 16.5 mg/kg, molecularly equivalent to MA (10 mg/kg).
- Group IV: receiving MA-PS; the final concentration of loaded MA was 1.13 mg/mL, molecularly equivalent to MA (10 mg/kg).
- Group V: receiving MefeGAL-PS; the final concentration of loaded MefeGAL was 2.45 mg/mL, molecularly equivalent to MA (10 mg/kg).
- Group VI: receiving Mix-PS; the estimated final concentration of loaded MA and MefeGAL was 1.66 and 2.26 mg/mL, respectively, molecularly equivalent to MA (10 mg/kg).

2.7. Anti-inflammatory activity

Acute anti-inflammatory activity was evaluated using carrageenan-induced mice hind paw edema assay described by D'D'Agostino et al. [50]. Initial paw volumes of all animals were measured using a plethysmometer apparatus (Ugo Basile, Milan, Italy) before treatment. Paw edema was induced by a subplantar injection of 50 μL of saline containing 1 % λ-carrageenan into the right hind paw. The oral treatment took place 1 h before the challenge with carrageenan. Paw volume was measured at different time intervals using a plethysmometer. The increase in paw volume was assessed as the difference between the paw volume measured at each time point and the basal paw volume measured immediately before λ-carrageenan injection.

2.8. Analgesic activity

Acetic acid-induced writhing test was performed as reported previously [51]. Each group formed by six mice as described above (group CTRL, group MA, group MefeGAL, group MA-PS, group MefeGAL-PS, group Mix-PS), was in turn divided into two subgroups. The mice were injected intraperitoneally with 0.6 % acetic acid (10 mL/kg body weight of the animal) 4 hours (the first subgroup) and 48 hours (the second subgroup) after drug treatment. Number of abdominal constrictions and extension of the trunk and hind limbs were counted for each group of mice starting from 5 minutes after the injection of acetic acid up to 20 minutes. The analgesic effect was expressed as number of writhing episodes compared to the control.

2.9. Ulcerogenicity studies

NSAID-induced gastric damage in mice was evaluated following the procedure described by Chan et al. [52]. Each group formed by six mice (group CTRL, group MA, group MefeGAL, group MA-PS, group MefeGAL-PS, group Mix-PS), was in turn divided into two subgroups as described above. The animals were fasted (16–18 h) prior to a single oral dose of the control, the test compounds, or their PSs; the mice belonging to three subgroups were euthanized after 4 h, while the others three 48 h later from treatment. After mouse euthanasia, the stomach was excised along its greater curvature and rinsed with normal saline. The gastric mucosa was then examined by means of a magnifying glass for the presence of irritation or frank haemorrhagic lesions (ulcers). No irritation was assigned a score of 0, irritations were scored as 0.5 and ulcerations were scored according to their length (a score of 1 for lesions with a length between 1 and 2 mm; a score of 2 for lesions with a length between 2 and 3 mm; a score of 3 for lesions greater than 3 mm). The sum of total scores was used for comparison.

2.10. Cell culture

Caco-2 human colorectal adenocarcinoma cells and A549 adenocarcinoma human alveolar basal epithelial cells were obtained from the American Type Tissue Collection (ATCC) and used at passages 35–40 and 30–35, respectively. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10 % (v/v)

Fetal Bovine Serum (FBS; Sigma-Aldrich), 0.1 mg/mL streptomycin, 100 units/mL penicillin, 0.25 μg/mL amphotericin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich) at 37°C with 5 % CO_2.

2.10.1. Cytotoxicity testing

PrestoBlue Cell Viability Reagent (Thermo-Fisher) was used to assess the metabolic activity of cells following treatments as an indication of cell killing. Cells were seeded at a density of 1 $\times 10^4$ cells per well in 96 well plates (Corning) for 24 hours prior to assaying. For this *in vitro* test, MA-PS, MefeGAL-PS and Mix-PS were resuspended in water and filtered (to remove possible bacteria contamination), and appropriately diluted to obtain the concentration range 0.01–10 mg/mL (total suspension including polymers and drugs). Treatments were applied to cells for 48 hours diluted in DMEM (no phenol red; Thermo-Fisher) supplemented as described above, however with the lack of antibiotics in the medium. Following exposure, treatment solutions were removed, cells were washed with PBS and 100 μL per well of 10 % PrestoBlue reagent diluted in DMEM (no phenol red) was applied to cells for 90 minutes. Solution fluorescence was then measured at 560/600 nm ($\lambda_{exc}/\lambda_{em}$), and relative metabolic activity calculated by setting the values of the negative control as 100 % and the positive control (1.0 % Triton X-100) as 0 %.

2.11. Statistical analysis

All analyses were conducted using Graph-Pad Prism (GraphPad Software Inc., San Diego, CA). The significance of differences between groups was determined by one or two-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test for multiple comparisons. In details, for the *in vivo* experiments, we used two-way ANOVA for Fig. 1 and a one-way ANOVA for Fig. 2 and Table 2. The level of statistical significance was * $p < 0.05$. Chi-square value (H) and degrees of freedom were included in Fig. 2.

3. Results and discussion

3.1. Polymer-(pro)drug solid dispersion (PS) formulation

MA, MefeGAL and a mixture of both were formulated into solid dispersions with the use of poly(glycerol adipate) PGA and Pluronic-F_{68} polymers. Here, as has been reported in previous literature examples [47,53], polymeric carriers have been used to enhance the aqueous solubility of water insoluble drugs *via* the formation and aqueous suspension of a solid polymer-drug dispersion. The raise in absorbance of the polymeric formulations compared to the unformulated (pro)drug when resuspended in water, ΔA %, was adopted as semiquantitative way to analyse water solubility improvement [47,54].

All the newly prepared formulations showed an enhancement of the apparent water solubility of the mefenamic-based (pro)drugs used (see Table 1). In particular, when a lower amount of the two polymers was used (formulations for *in vitro* tests) in more diluted circumstances, a slightly higher ΔA % was observed. This could be attributed to possible enhanced polymer-drug interactions which may decrease in favour of more polymer(1)-polymer(2) interactions in the *in vivo* formulations (presenting a higher amount in polymers). Finally, due to absorbance overlapping in the spectra of the prodrug and drug, the ΔA % of the Mix

	Treatment					
Time	CTRL	MA	MefeGAL	MA-Ps	MefeGAL-Ps	Mix-Ps
2h	0.767 ± 0.056	0.467 ± 0.056 *	0.733 ± 0.049	0.633 ± 0.021	0.683 ± 0.031	0.600 ± 0.037
4h	0.983 ± 0.070	0.700 ± 0.026	0.883 ± 0.048	0.683 ± 0.054	0.783 ± 0.070	0.633 ± 0.042 °
6h	0.783 ± 0.098	0.683 ± 0.048	0.750 ± 0.096	0.650 ± 0.043	0.667 ± 0.056	0.567 ± 0.042
24h	0.783 ± 0.060	0.667 ± 0.033	0.467 ± 0.042 +	0.533 ± 0.042	0.500 ± 0.026 §	0.467 ± 0.021 °
48h	0.767 ± 0.056	0.683 ± 0.048	0.467 ± 0.061 +	0.517 ± 0.031 #	0.467 ± 0.049 §	0.500 ± 0.052 °
72h	0.917 ± 0.087	0.917 ± 0.040	0.767 ± 0.099	0.916 ± 0.042	0.800 ± 0.077	0.802 ± 0.079

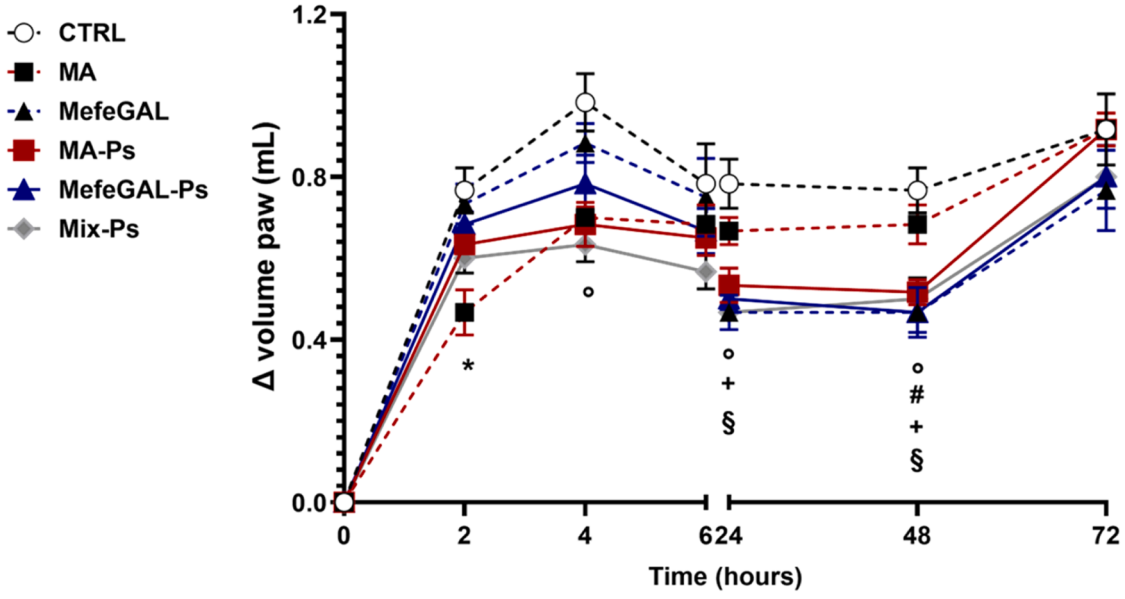


Fig. 1. Mouse paw edema. Effect of oral administration of MA, MefeGAL, MA-PS, MefeGAL-PS, Mix-PS on carrageenan-induced hyperalgesia in mice paws evaluated at 2, 4, 6, 24, 48 and 72 h after λ-carrageenan challenge. The increase in paw volume was evaluated and is expressed as the difference in paw volume measured at each time point and the basal paw volume measured immediately before λ-carrageenan injection. Data in the table are expressed as means ± SEM for each group (n = 6). *$p<0.05$ MA vs CTRL; °$p<0.05$ Mix-PS vs CTRL; #$p<0.05$ MA-PS vs CTRL; +$p<0.05$ MefeGAL vs CTRL; §$p<0.05$ MefeGAL-PS vs CTRL.

	Treatment					
Time	CTRL	MA	MefeGAL	MA-Ps	MefeGAL-Ps	MIX-Ps
4h	38.33 ± 1.706	16.83 ± 0.946 ***	29.17 ± 2.414	41.17 ± 2.774	29.50 ± 1.432	24.83 ± 1.922*
48h	38.50 ± 2.717	30.17 ± 3.619	23.33 ± 1.838**	26.33 ± 3.242*	23.33 ± 2.445**	24.67 ± 2.376**

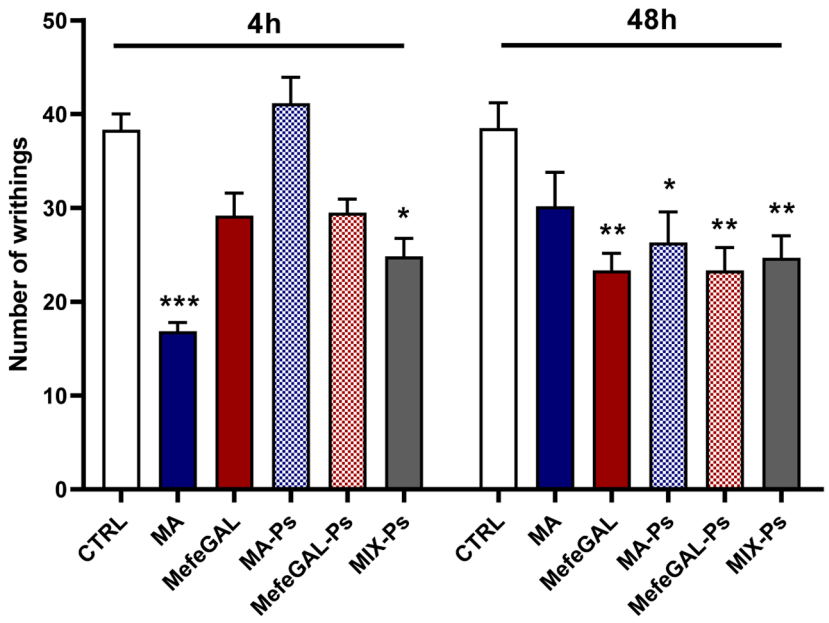


Fig. 2. Total number of writhing episodes within 20 minutes after acetic acid injection. Effect of oral administration of MA, MefeGAL, MA-PS, MefeGAL-PS, Mix-PS at 4 h ($H = 20.23$, $df = 25$; $p = 0.734$) and 48 h ($H = 39.97$, $df = 25$; $p = 0.0294$) after administration of 0.6 % acetic acid (10 mL/kg). Data are expressed as means ± SEM for each group ($n = 6$). *$p<0.05$. **$p<0.01$ and ***$p<0.001$ vs CTRL.

Table 1
MA and MefeGAL apparent water solubility enhancement, when formulated into PS, reported as ΔA % at 292 nm (MA detection) and 354 nm (MefeGaL detection).

		ΔA % (292 nm)	ΔA % (354 nm)
in vitro	MA-PS	68.7	–
	MefeGAL-PS	–	67.2
in vivo	MA-PS	39.3	–
	MefeGAL-PS	–	54.7

*for *in vitro* and *in vivo* experiments the amount of polymers used varied (see materials and methods section).

was not reported. However, the efficacy in bringing the drugs in solutions of these formulations has been confirmed by the enhanced performance in all the biological tests.

3.2. Effect of PS on Carrageenan-induced paw edema

To evaluate the potential *in vivo* anti-inflammatory profile exerted by the resuspended (pro)drug-PS formulations when administered orally, the conventional carrageenan-induced rat paw edema assay was performed. MA was orally administered at the dose of 10 mg/kg and was able to inhibit paw edema formation during the first phase (0–6 h); in particular, the anti-inflammatory activity was significative at 2 h after λ-carrageenan injection as shown in Fig. 1 (two-way ANOVA, effect of interaction, $F_{30,180} = 2.312$; $p<0.05$). During the second phase (24–72 h), it did not show any significative effect while its polymeric formulation (MA-PS) showed a reduced paw edema at 48 h ($p<0.05$). On the other hand, when we injected equimolecular dose of MefeGAL (16.5 mg/kg) or MefeGAL-PS, no anti-inflammatory effects were observed during the first phase of the test (0–6 h), while produced a significant activity during the second phase (at 24–48 h; $p<0.05$) confirming the delayed pharmacological time-profile typical of the galactosylated prodrugs of NSAIDs [5–9]. Surprisingly, Mix-PS was able to decrease in paw edema formation during both phases displaying a significant anti-inflammatory effect at 4 h ($p<0.05$), at 24 h ($p<0.05$) and 48 h ($p<0.05$) after λ-carrageenan injection (Fig. 1). Although at 6 hours the statistical analysis was not significant for the Mix-PS group compared with the CTRL group, there is a decrease in mouse paw edema formation confirming the anti-inflammatory activity ($p = 0.4129$). Simultaneous application of the galactosylated prodrug approach and particulate drug delivery system combined with a multicomponent strategy (parent drug and prodrug in the same PS formulation), demonstrated a highly desirable prolonged anti-inflammatory effect, but with a much more rapid onset of action than MefeGAL or the respective PS formulation (MefeGAL-PS). Such a pharmacological profile would bring numerous advantages: sustained release of an NSAID at a slow rate over a prolonged period would reduce dosing frequency, eliminate the use of PPIs that are co-prescribed during chronic NSAID therapies but at the same time ensure pharmacological activity as early as the first few hours after oral treatment by increasing patient compliance.

3.3. Effect of PS on acetic acid-induced writhing test

Analgesic activity was carried out by evaluating the number of abdominal stretching induced by acetic acid at preselected time points, as determined by previous test. The results are reported in Fig. 2 (one-way ANOVA, effect of interaction, $F_{11,60} = 9.197$; $p<0.0001$). Mice were injected intraperitoneally with 0.6 % acetic acid (10 mL/kg) 4 and 48 hours after oral drug treatment. Confirming the same trend observed for anti-inflammatory performances, MA at the dose of 10 mg/kg produced a very significant reduction ($p<0.001$) in writhing number at 4 h from oral administration as well as Mix-PS ($p<0.01$) compared with CTRL group. Conversely, MA-PS, MefeGAL and its respective PS (MefeGAL-PS) showed such activity after 48 h ($p<0.05$ and $p<0.01$

respectively). This response at different times is probably due to hydrolytic release of active drug MA from its galactosylated prodrug MefeGAL as well as a controlled release of MA or MefeGAL from their developed PS formulations and thus corroborating the new pharmacological time-profile that MA takes on when it is conjugated to galactose or formulated with PGA and Pluronic® F_{68} based-PS. The development of a solid dispersion drug delivery system based-MefeGAL also proved to be a viable strategy in terms of analgesic potential, because it would mean having an active drug capable of covering a 44-hour time window. Thus, while individual release strategies ensure a delayed and prolonged MA profile, Mix-PS would be able to ensure pharmacological activity as early as the first hours of intake.

Considering the amphiphilic nature of the polymers and the hydrophobic nature of the drugs, the possible formation of nanoaggregates of the PS formulations when resuspended in water might be another factor affecting the delayed activity. This could be due to the sustained release of drugs from the formulations, which may affect the drugs' pharmacokinetics and minimise or delay the side effect behaviours. As preliminary test, nanoaggregates of different sizes, and different distribution size profiles (monomodal and bimodal) were observed by DLS analysis (Figure S1 see Supporting Information section).

3.4. Effect of PS on ulcerogenic activity

To complete the *in vivo* study, the potential gastric toxic effects exerted at prefixed times by the tested polymer-(pro)drug formulations using an acute NSAID-induced ulcerogenic assay. Ulcerogenic property is expressed by an individual score assigned based on irritation/haemorrhagic lesions (ulcers) in gastric mucosa, as reported in legend. Each score corresponds to a different severity of gastric damage. The study of ulcerogenic activity reported in Table 2 (one-way ANOVA, effect of interaction, $F_{11,60} = 12.29$, $p<0.0001$) indicates that the parent drug (MA), which has the free carboxylic group, showed significant ulcerogenic property at 4 h and 48 h. The galactosylated derivative (MefeGAL) displayed a lower gastric injury score than MA and only at 48 h from oral administration. This was in agreement with the controlled hydrolysis that MefeGAL underwent *in vivo*, leading to the release of the active drug. i.e., MA, which, in turn, caused a mild irritant effect at the gastric level. By applying the galactosylated prodrug-based polymeric PS strategy, not even the irritating effect at 48 h was found on the gastric mucosa confirming the capability of resetting gastrointestinal adverse events when galactose and polymers are used as carrier systems for NSAIDs.

No damage was observed 4 h after oral administration of MA-PS and Mix-PS, as the local contact of the carboxyl group of MA with the gastric mucosa was momentarily masked by the presence of the polymeric scaffold of which the PS were composed. In contrast, 48 h after oral administration of MA-PS, the presence of 1-mm long gastric ulcers was detected while Mix-PS did not cause ulcerogenic activity but only irritative effect.

Table 2
Ulcerogenic activity of MA, MefeGAL, MA-PS, MefeGAL-PS, Mix-PS 4 and 48 h after treatment. [a]A score of 0.5 was assigned for irritation; a score of 1 was assigned for lesions with a length between 1- and 2-mm. Data are expressed as means for each group (n = 6). Significance of MA versus Control: ****$p<0.0001$; MA-PS and Mix-PS versus MA: $^{\#\#\#\#}p<0.0001$.

Compounds	Gastric Lesion Score (4 h)[a]	Gastric Lesion Score (48 h)[a]
Control (CMC 0.5 %)	0	0
MA	1****	1****
MefeGAL	0	0.5
MA-PS	$0^{\#\#\#\#}$	1
MefeGAL-PS	0	0
Mix-PS	$0^{\#\#\#\#}$	0.5

3.5. In vitro cytotoxic effect

The cytotoxicity of MA, MefeGAL and their formulations, MA-PS and MefeGAL-PS, were tested on colorectal adenocarcinoma Caco-2 cells and non-small cell lung carcinoma A549 cells (Fig. 3A-B). All treatments induced concentration-dependent toxicity to both cell lines, as indicated by dose-response decline in cellular metabolic activity, highlighting their anticancer activity (Fig. 3). Calculation of resulting IC_{50} values (half maximal inhibitory concentrations) was performed to enable subsequent comparison between the treatments (Fig. 3C). It is noted that the potency of MA calculated in the current study is in a comparable micromolar range and thus in agreement with previously reported IC50 values in human lung carcinoma A549 and colonic carcinoma cells [55–58]. *In vitro* testing reveals that the prodrug MefeGAL demonstrates significantly higher potencies than MA; with MefeGAL inducing a 4.6-fold increase in potency in Caco-2 intestinal cells ($p < 0.001$) and 2.7-fold increase in A549 lung cells ($p < 0.001$) relative to MA treatment. Thus, the galactosylated prodrug approached appears a potent means of increasing the cytotoxicity of MA.

Application of PS formulations treatments in general induced slight increases in potency at reducing cellular metabolic activity relative to the unformulated counterparts. In Caco-2 cells, MA-PS treatment induced a statistically significant 1.2-fold increase in potency relative to MA treatment ($p < 0.01$). However, in A549 cells a slight, but non-significant decrease in potency was observed between MA-PS and MA treatments. MefeGAL-PS generated increased cytotoxicity relative to unformulated MefeGAL treatment, with IC_{50} values 1.2-fold lower in both Caco-2 and A549, however the differences in potency values were determined non-significant.

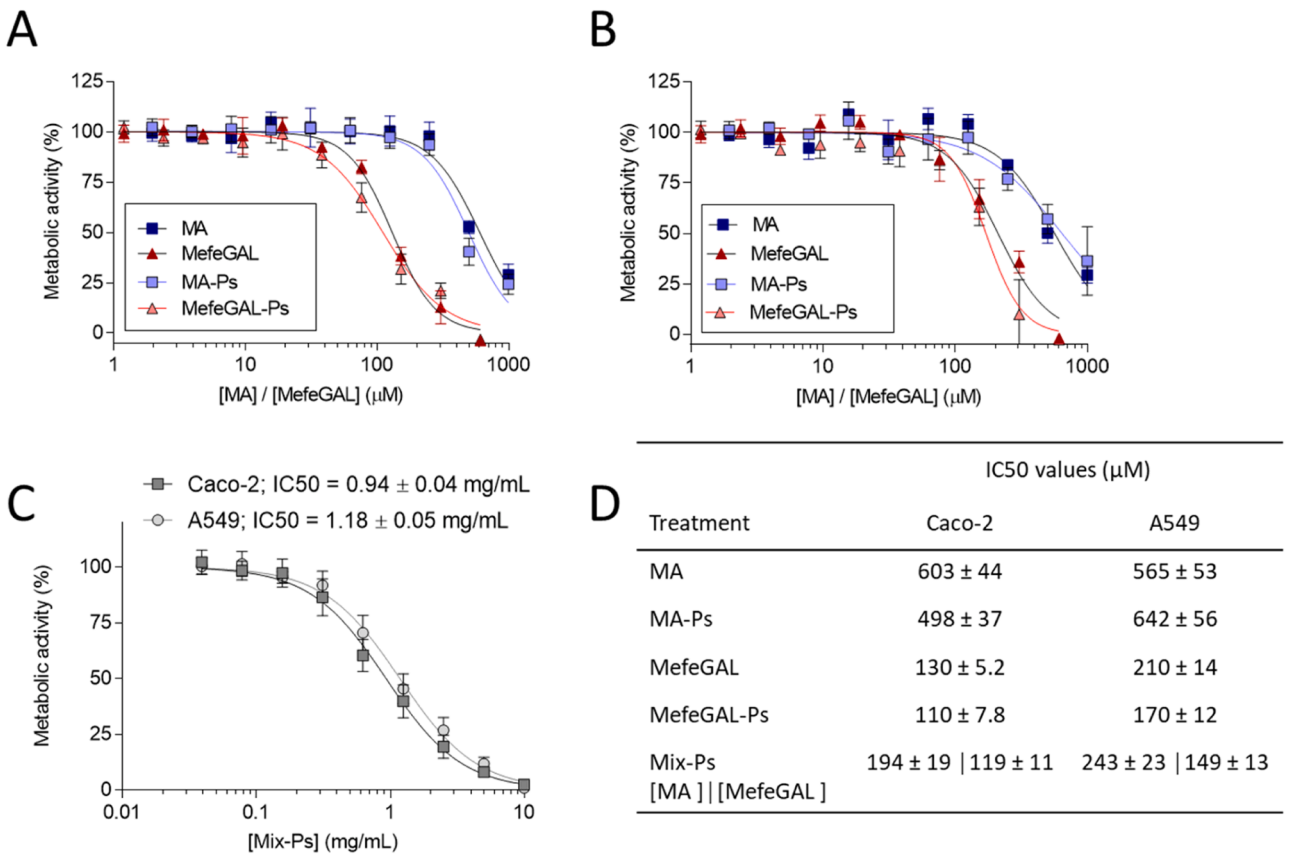
Finally, *in vitro* testing of polymeric formulations of mixed MA/MefeGAL (Mix-PS) was performed (Fig. 3C). IC_{50} values were calculated based on formulation concentration (mg/mL) and subsequent determination of MA and MefeGAL IC_{50} concentrations based on drug loading within the formulation was performed (Fig. 3D). Mix-PS demonstrated higher potency on both Caco-2 and A549 cells relative to the MA-PS IC_{50} concentrations and comparable IC_{50} values when MefeGAL-PS concentrations are considered. The later observation indicates that the MefeGAL present in the formulation is likely driving the anticancer activity of the Mix-PS formulation treatment.

Together the *in vitro* data highlight that MA demonstrates potent cytotoxicity in colorectal and non-small cell lung cancer, and that this activity can be substantially increased through the use of a galactosylated prodrug approach (MefeGAL). Moreover, polymeric solid dispersions of poly(glycerol adipate) (PGA) and Pluronic® F_{68}, which may be beneficial in enhancing the *in vivo* bioavailability and pharmacokinetic properties of MA and MefeGAL, does not impede the observed antiproliferative activity of the compounds. Therefore, MefeGAL formulated with polymeric solid dispersions presents as a treatment with potential antitumour activity, with increased aqueous solubility and thus warrants future investigation.

4. Conclusions

The numerous advantages offered by the galactose carrier did not always overcome all the shortcomings of the drug of interest as in the case of MA. Therefore, to reduce its adverse gastric effects, the galactosylated prodrug approach was applied in tandem with a polymeric solid dispersion formulation strategy using PGA and a PEG-based surfactant.

The versatile polymer-(pro)drug material could be prepared as solid film for oral administration that demonstrate anti-inflammatory and analgesic activity, or as nanoaggregates possessing cytotoxic effects suitable for intravenous administration. Comparing the *in vivo* biological performance of MefeGAL-PS with those of MA, MefeGAL and MA-PS, it was seen that the new polymer-MefeGAL delivery system showed the same prolonged and delayed analgesic and anti-inflammatory profile as



D

Treatment	IC50 values (μM) Caco-2	A549
MA	603 ± 44	565 ± 53
MA-Ps	498 ± 37	642 ± 56
MefeGAL	130 ± 5.2	210 ± 14
MefeGAL-Ps	110 ± 7.8	170 ± 12
Mix-Ps [MA] | [MefeGAL]	194 ± 19 | 119 ± 11	243 ± 23 | 149 ± 13

Fig. 3. Cytotoxicity of compounds and formulations in (A) Caco-2 intestinal cells and (B) A549 lung cells. (C) Testing of Mix-PS on Caco-2 and A549 cells. Treatments were applied for 48 h, and cellular metabolic activity evaluated with PrestoBlue assay. Data presented as mean ± SD and comes from three independent experiments (n = 3). (D) IC_{50} values calculated from nonlinear dose response fitting and values presented as mean ± SEM. Mix-PS IC_{50} values calculated show the MA and MefeGAL concentrations presented aside each other (MA | MefeGAL).

MefeGAL but did not cause any gastrointestinal irritation. Thus, we can infer that the combination of multiple drug delivery systems improved the physicochemical, pharmacological, and toxicological profile of MA.

A further solid dispersion, Mix-PS, was developed containing both MA and MefeGAL solid film form. While the single-drug release strategies (MA-PS and MefeGAL-PS) ensured a delayed and sustained pharmacological profile, Mix-PS pharmacological effect present from the first hours after administration, guaranteeing a time duration of approximately 44 hours with only minor irritation of the gastric mucosa.

Evaluation of antiproliferative activity indicated MefeGAL-PS induce a non-significant increase in cytotoxicity compared to the treatment with unformulated MefeGAL. Notably, treatment with Mix-PS presented with statistically significant higher cytotoxicity than MA-PS and MefeGAL-PS counterparts in *in vitro* models of intestinal and lung cancer models.

The preliminary data reported in this work are promising evidence that supports the potentiality of the combination of two formulation strategies (prodrug and polymeric solid dispersion) as new drug delivery route for improving solubility and biological activity of NSAIDs. However, as a preliminary and proof-of-concept work, further experimental efforts, e.g. regarding the *in vivo* mode of action in vivo and comparison with other strategies, will be required to assess the advantages and limitations of the proposed new method.

CRediT authorship contribution statement

Fabrizio Maria Liguori: Investigation, Formal analysis. **Roberto Russo:** Writing – review & editing, Supervision, Project administration. **Barbara Rolando:** Formal analysis, Data curation. **Mariarosaria Cuozzo:** Investigation, Formal analysis, Data curation. **Marica Erminia Schiano:** Investigation, Formal analysis. **Philippa L. Jacob:** Methodology, Formal analysis, Data curation. **Emily Dixon:** Formal analysis, Investigation. **Eleni Axioti:** Methodology, Formal analysis. **Cara Moloney:** Methodology, Formal analysis. **Maria Grazia Rimoli:** Writing – review & editing, Supervision, Project administration. **Valentina Cuzzuzoli Crucitti:** Investigation, Formal analysis. **Federica Sodano:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Conceptualization. **Robert J. Cavanagh:** Writing – original draft, Investigation, Formal analysis. **Vincenzo Taresco:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Claudia Cristiano:** Writing – original draft, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of competing interest

The authors declare no competing financial interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116647.

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