

Supplementary Information

Dexamethasone palmitate nanoparticles: an efficient and safe treatment for rheumatoid arthritis.

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Formulation

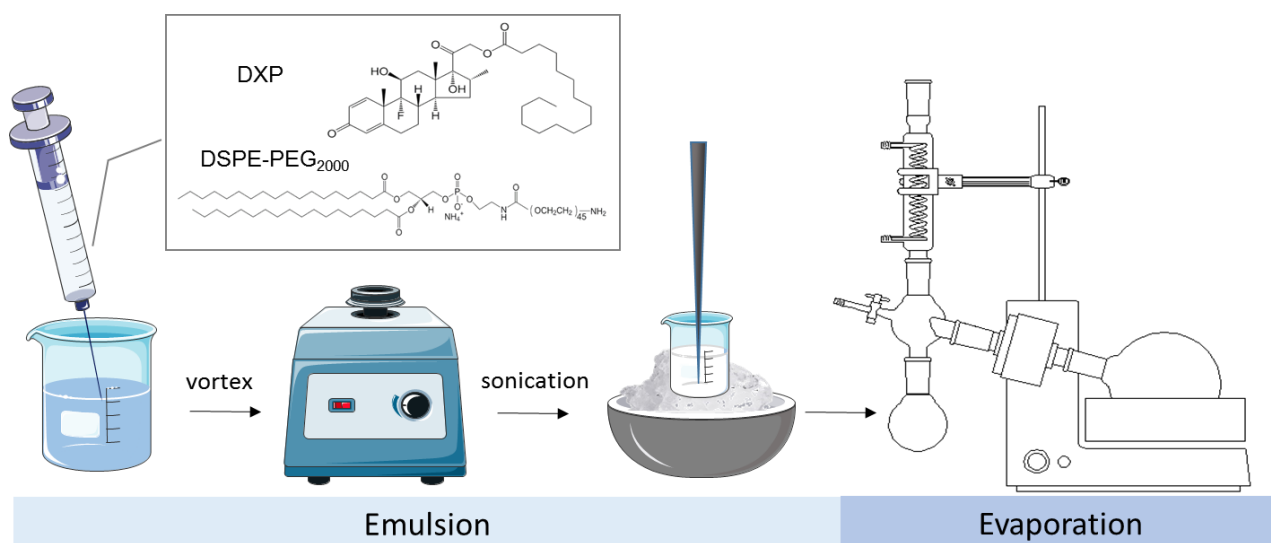


Fig. S1: Scheme of DXP-NPs preparation.

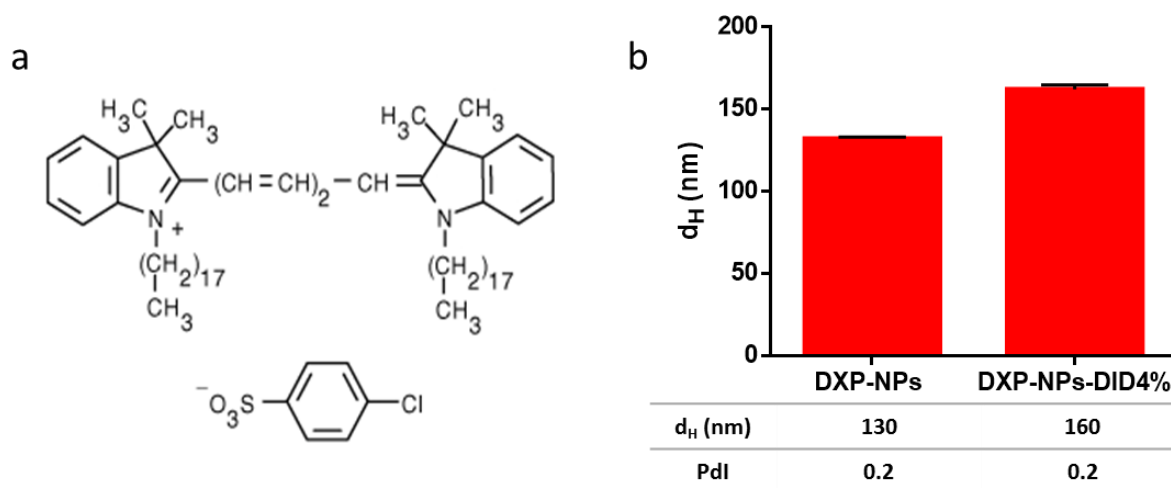


Fig. S2: **a.** Chemical formula of fluorescent DID. **b.** Size and Pdl of fluorescent DXP-NPs compared to plain DXP-NPs ($n=3$ independent formulations).

DXP-NP characterization

DXP-NPs were characterized for their hydrodynamic diameter, polydispersity index (Pdl) and zeta potential with DLS measurement using a Malvern Zetasizer NanoZS 6.12 (173° scattering angle, 25°C). Before measurement, samples were brought back to room temperature. For size and Pdl measurement, samples were diluted at 1/10 in milliQ water and a good attenuator value of 6 was obtained. The mean diameter and Pdl values corresponded to the average of three measurements done on the same sample in automatic mode with a general-purpose analysis. Zeta potential was measured after 1/10 dilution in NaCl 1mM solution. The zeta potential values corresponded to the average of three measurements done on the same sample in automatic mode with a Smoluchowski analysis.

Sample preparation for X-ray diffraction

DXP-NPs were prepared as described in material and methods. The day of preparation or 3 weeks after storage at 4°C, nanoparticles were concentrated using Amicon 100kDa filter. 0.5mL of the suspension was added inside the Amicon device and centrifuge 10min at 13400 rpm using a minispin centrifuge (Eppendorf). Concentrated DXP-NPs were recovered from the top of the filter and gently introduced into quartz capillaries before sealing them. X-ray powder diffraction (XRPD) measurements were performed using a Rigaku rotating copper anode automated diffractometer operating at 50kV and 200mA using Cu K α radiation. The intensity of the diffracted X-ray beam was measured as a function of the theta angle (1 to 60°).

Sample preparation for TEM and Cryo-TEM

Transmission electron microscopy (TEM) and cryo-TEM was performed at I2BC (CNRS, Gif-sur-Yvette, France). For TEM, a volume of 5 μ L of the nanoparticle suspension at 5mg/mL DXP was deposited for 1 minute on a glow discharged 400 mesh formwar-coated copper grid. Negative staining was performed by addition of a drop of uranyl acetate at 2% w/w for 30 seconds. Excess solution was removed and grids were left to dry before observation. The observations were carried out on a JEOL JEM-1400 microscope at an acceleration voltage of 80kV, images were acquired using an Orius camera (Gatan Inc, USA).

For cryo-TEM, a volume of 5 μ L of the DXP-NP suspension at 5mg/ml DXP was deposited on glow-discharged Lacey copper grids covered with carbon film containing holes. The excess solution was blotted off for 4 seconds on a filter paper and the residual thin film was vitrified after immersion in liquid ethane using a Leica EM GP automatic system (Leica, Austria) under a 90% humidity atmosphere. The sample was transferred using liquid nitrogen to a cryo-specimen holder and observed using a JEOL JEM-1400 microscope at an acceleration voltage of 120kV and a defocus of -6 μ m. Images were acquired using an Orius camera (Gatan Inc, USA).

Encapsulation efficiency

After DXP nanoparticles (DXP-NPs) preparation, quantification of encapsulated DXP and DSPE-PEG₂₀₀₀ was performed with HPLC coupled to an evaporative light-scattering detector (ELSD, Eurosep, Cergy, France) and a UV detector (785A, Applied Biosystems). DXP was detected with both detectors and DSPE-PEG₂₀₀₀ was only detected with ELSD. UV detection was performed at 240nm and ELSD detection settings were a nebulization temperature of 35°C and an evaporation temperature of 45°C. An autosampler (Series 200, Perkin Elmer) was employed connected to a Perkin Elmer pump and to both detectors UV and ELSD. The SymmetryShieldTM RP18 column (5 μ m, 250 \times 4.6 mm; Waters, France) was maintained at room temperature, the mobile phase was composed of MeOH:ACN:Ammonium acetate (pH 4.00, 200mM) 70:20:10 added with 0.043% acetic acid and 0.104% trimethylamine, flow rate was set at 1mL/min.

To separate DXP-NPs from non-encapsulated molecules, ultracentrifugation was performed at 40000rpm (=109760g) for 4h at 4°C (Beckman Coulter Optima LE-80K ultracentrifuge, 70-1Ti rotor). The supernatant was delicately removed to avoid resuspension of the pellet and freeze-dried for 24h (Alpha 1-2 LD Plus, Bioblock, France) after freezing in liquid nitrogen (-196°C). Lyophilisates were dissolved in methanol to reach suitable concentrations within the range of the calibration curve. A volume of 30 μ L of sample dissolved in MeOH was injected and analyzed during 12min. Retention times were 7min and 9min for DXP and DSPE-PEG₂₀₀₀, respectively. The concentration ranges of the

calibration curves were 12.5-750 μ g/mL for DXP and 37.5-375 μ g/mL for DSPE-PEG₂₀₀₀. ELSD detection calibration curves followed a power law model, equations and correlation coefficients were $y=219.71x^{1.2458}$, $R^2=0.9959$ for DXP and $y=144.12x^{1.2714}$, $R^2=0.9991$ for DSPE-PEG₂₀₀₀, respectively. UV calibration curve of DXP followed a linear model, $y=486.2x+4.6095$, $R^2=0.9987$. Encapsulation efficiency was calculated as the DXP or DSPE-PEG₂₀₀₀ amount encapsulated compared to the initial amount weighted for the DXP-NPs preparation. Drug loading corresponds to the proportion of equivalent DXM mass encapsulated compared to the total mass of the DXP-NPs.

CIA model

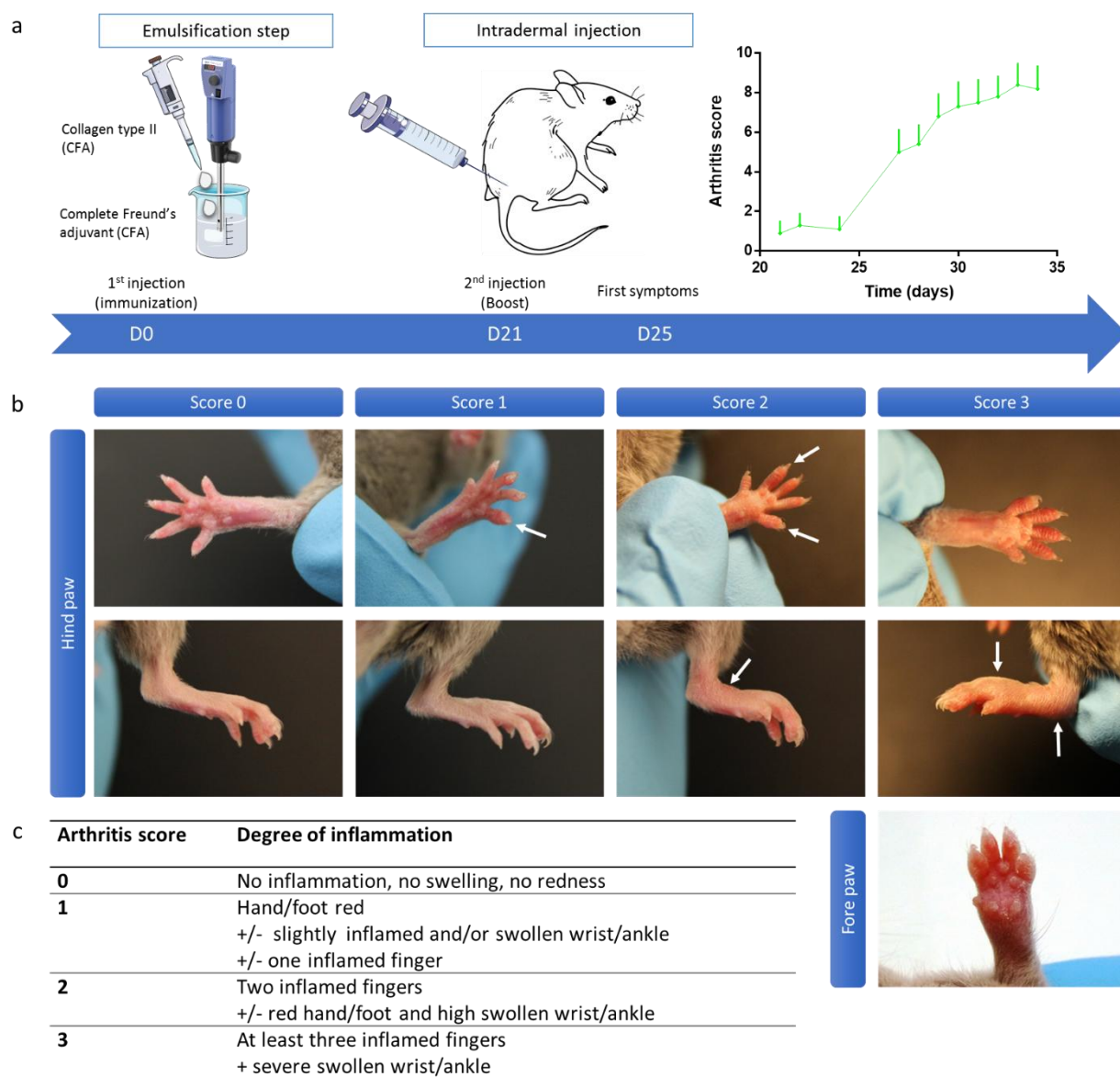


Fig. S3: **a.** Schematic representation of the CIA protocol. The first step corresponds to the emulsification of type II collagen with CFA using an Ultraturrax. Emulsion is then injected intradermally to DBA/1 males on days 0 and 21. **b.** Arthritis first signs appeared 25 days after the first immunization step. **c.** Hind paws and fore paws pictures based on inflammation visual score described in table

In vivo imaging

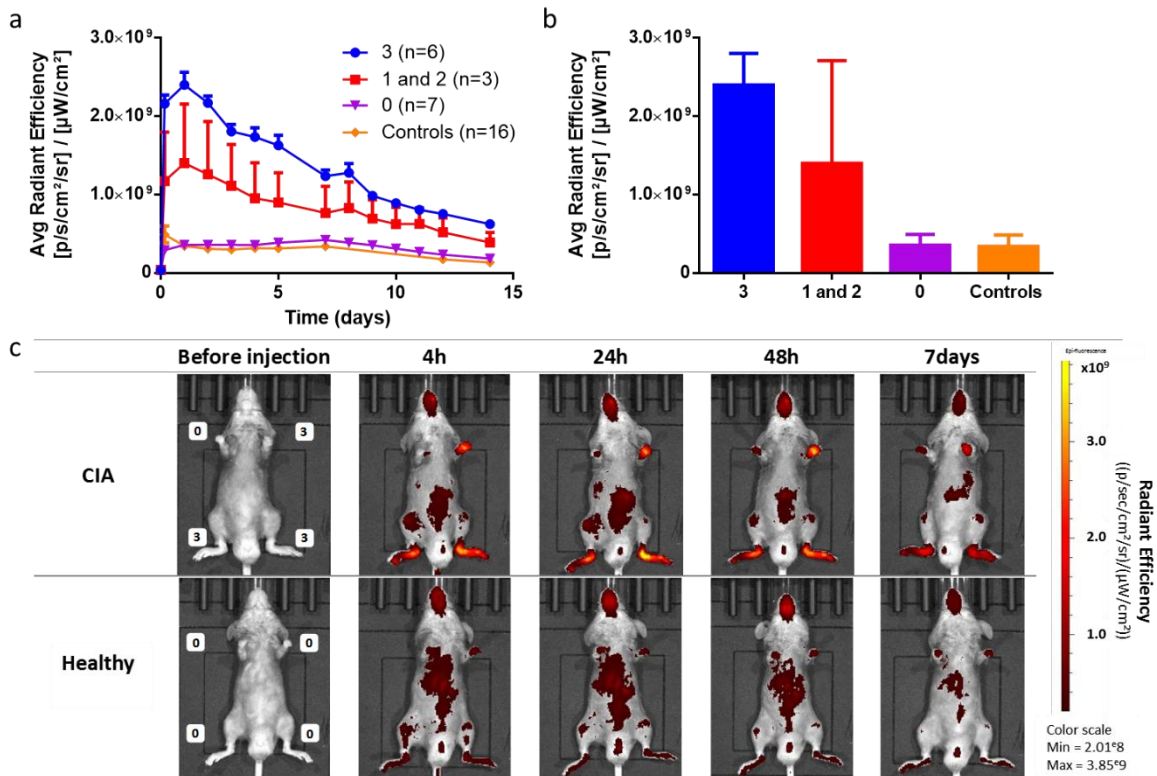


Fig. S4: Accumulation of DXP-NPs in inflamed joints, impact of arthritis severity. After IV injection of fluorescent nanoparticles, the evolution of the NIR signal (ventral view) in healthy mice (n=4) and CIA mice (n=4) was monitored according to the arthritis score on each paw. **a.** Fluorescence signal in mice paws for 14 days. Paws of healthy mice (yellow diamonds) and score 0 paws of CIA mice (purple triangles) displays the same basal level of fluorescence, scores 1 and 2 paws of CIA mice presented an increase in fluorescence signal above the auto-fluorescence level up to 48h after injection followed by a slow decrease down to basal level after 14 days. Highly inflamed paws (score 3, blue dots) displayed a strong fluorescence signal with a maximal level 24h after injection. **b.** Fluorescence signal in paws 24h after IV injection. Nanoparticles accumulation in highly inflamed paws (score 3, blue) was significantly higher than mildly inflamed paws (scores 1 and 2, red) and regular paws (score 1, purple/ healthy, yellow). The strongest joint inflammation, the highest nanoparticles accumulation due to the EPR effect. **c.** In vivo representative images of one mouse per group before and after injection up to 7 days. Scores of each paw are indicated in white squares on the “before injection” image.

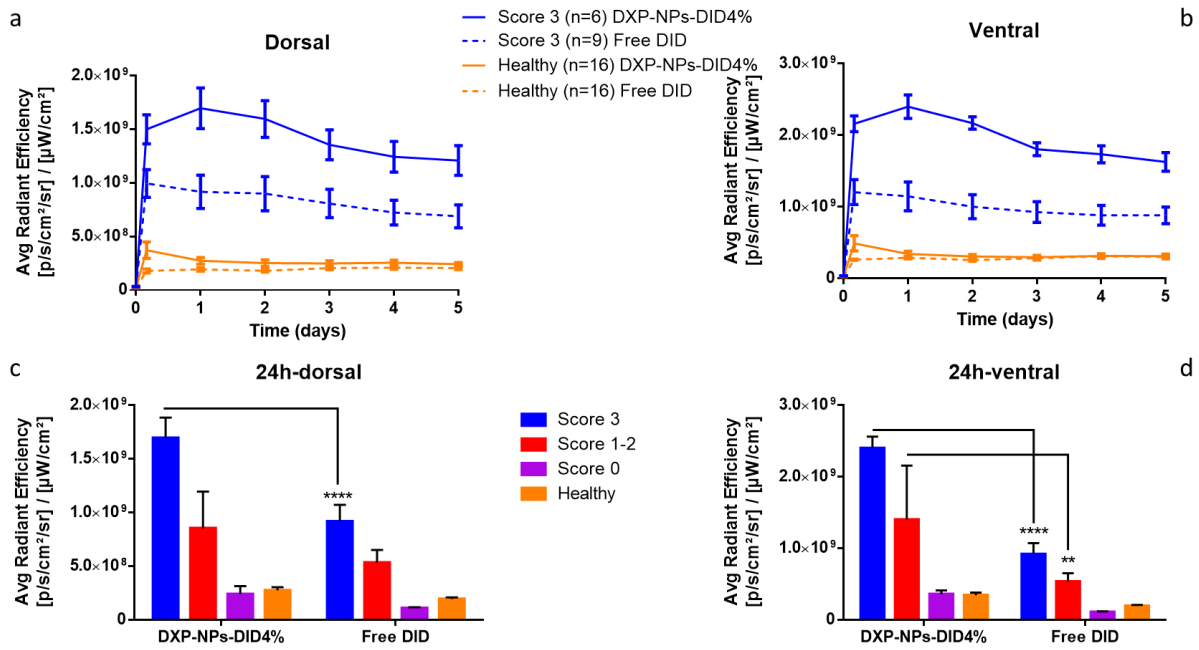


Fig. S5: *In vivo* joint detection of free DID compared to DXP-NPs-DID4%. After IV injection of a DID solution at the same DID concentration as DXP-NPs-DID4%, the fluorescent signal was quantified in CIA (n=6) and healthy mice (n=4). Dorsal and ventral pictures were taken. Evolution of the fluorescence signal on the dorsal (**a**) and ventral (**b**) positions after injection of free DID to healthy mice (dotted orange line) (n=4 mice, n=16 paws) and to CIA mice (dotted blue line) (n=6 mice, n=9 paws with score = 3) or DXP-NPs-DID4% to healthy mice (solid orange line) (n=4 mice, n=16 paws) and to CIA mice (solid blue line) (n=6 mice, n=6 paws with score = 3). Fluorescence signal at 24h measured in the dorsal (**c**) and ventral (**d**) images. Fluorescence signal in mice paws was recorded for 14 days on. Non-inflamed paws of healthy mice injected with DXP-NPs-DID4% and injected with free DID presented a similar low level of fluorescence. The fluorescence signal detected in highly inflamed paws of CIA mice (arthritis score 3) injected with DXP-NPs-DID4% (solid blue line) was twice as high as free DID (dotted blue line). 24h after the injection, pictures displayed the same pattern. On inflamed paws (arthritis scores of 3, 1 and 2) the fluorescent signal was significantly higher for DXP-NPs-DID4% compared to free DID. These results confirmed the specific accumulation of nanoparticles in inflamed joints.

Adverse events

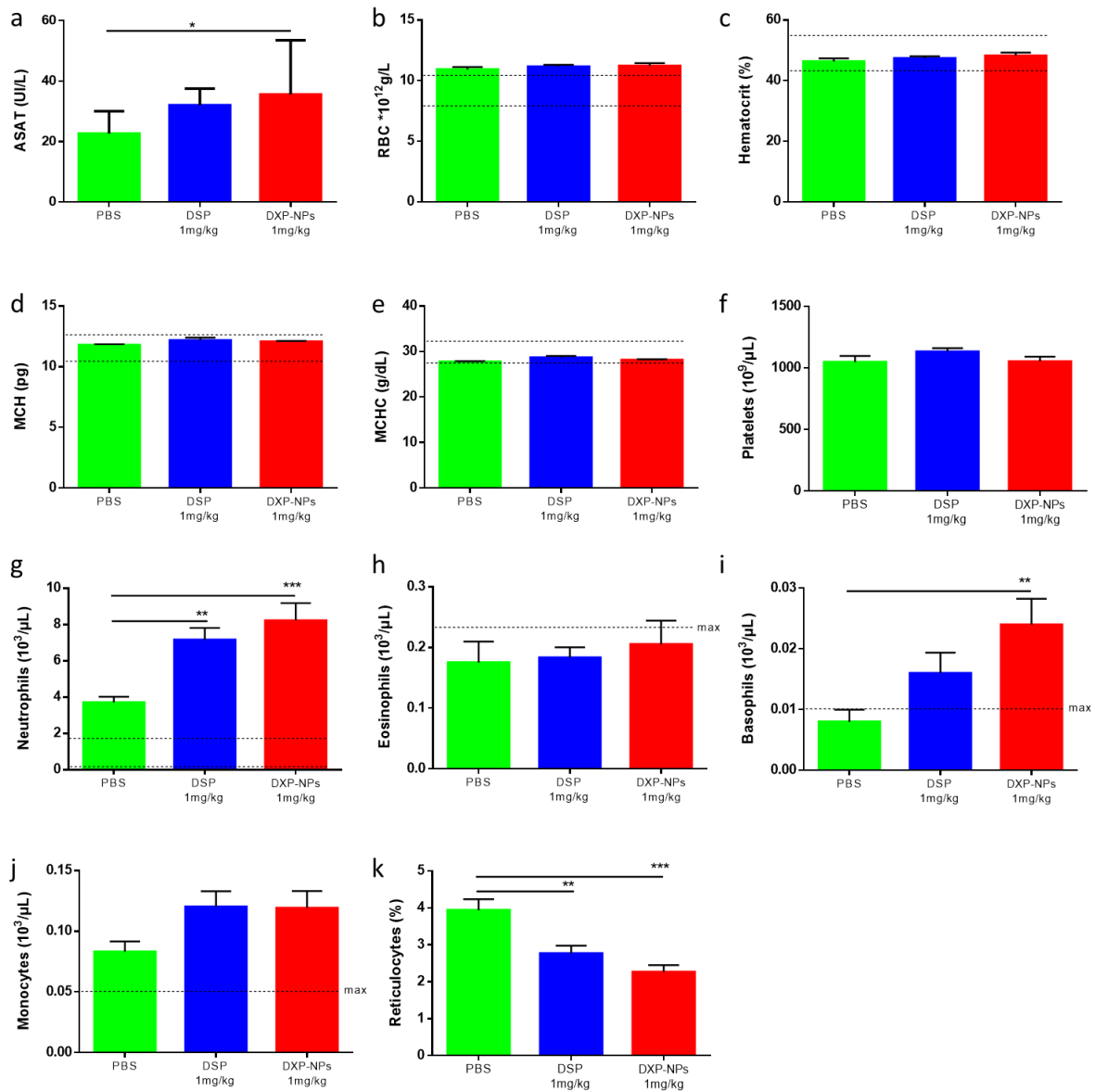


Fig. S6: Absence of adverse effects observed after DXP-NPs administration compared to DSP. Three groups of CIA mice (n=10) received PBS, or DSP 1mg/kg (eq.DXM) or DXP-NPs 1mg/kg (eq.DXM) on days 32, 34 and 36 after CIA induction. Mice were followed up to sacrifice on day 37, several biological parameters were analyzed. **a.** An increase of ASAT plasmatic concentration was detected for after DXP-NPs treatment compared to DSP and PBS. Red blood cells, hematocrit, MCH, MCHC and platelets (**b, c, d, e, f**) presented similar blood levels for the three treatment groups. An increase of neutrophils (**g**) and monocytes (**j**) was detected in the three groups. **h.** No modification of eosinophil concentration was detected for all groups. **i.** The blood basophils were increased with the administration of dexamethasone and a significant difference was observed between DXP-NPs and PBS. **k.** A diminution of the reticulocytes was detected for both DSP and DXP-NPs compared to PBS. For hematological parameters analyzed on total blood, dotted lines on graphs represent standard values for healthy mice. Results are presented as mean ± SEM (n=10). Statistical analysis was performed with One-Way ANOVA followed by Tukey's post-test (*p<0.05, **p<0.01, ***p<0.001).

Liver distribution of DXP and DXM

Liver distribution was carried out on DBA/10IaHsd mice. Experiments were approved by the ethical committee No 026 and by the French ministry of education and research (Accepted protocol No 2842-2015110914248481_v5). 9-12-week-old male DBA/10IaHsd mice were purchased from Envigo (UK) and let for one week after shipping for adaptation before starting experiments. The mice were kept in a separate animal room under climate controlled conditions with a 12h light/dark cycle, housed in polystyrene cages containing wood shavings and fed standard rodent chow and water *ad libitum*. Mouse colonies were screened and determined to be pathogen-free.

After intravenous administration, mice were euthanized and liver was taken off and homogenized in PBS using a micro-pestle coupled with a turbine at 2038rpm during the time needed to obtain a liquid preparation, approximately 5min. DXM and DXP were extracted from the homogenate using 100 μ g of homogenate. 100 μ L of internal standard mixture TestD and DXA at 4 μ g/mL was added and vortexed 30sec, followed by the addition of 3mL of 9/1 chloroform/methanol (v/v), vortexed for 3min and centrifuged in the same conditions as above. Organic phase was evaporated and 200 μ L acetonitrile was added and mixed during 1.5min. This final sample was analyzed by HPLC-UV. A Waters 717 Plus autosampler chromatographic system was employed equipped with a Waters 1525 binary HPLC pump, a Waters 2487 dual λ absorbance UV detector, and a Breeze software. The analysis was performed at 240nm wavelength using a SymmetryShieldTM RP18 column (5 μ m, 250 \times 4.6 mm; Waters, Saint-Quentin-en-Yvelines, France). The column temperature was maintained at 40°C. The mobile phase was composed by a mixture of acetonitrile and milliQ water: 85/15 v/v for DXP and 35/65 v/v for DXM. The mobile phase flow rate was 1.2mL/min, the injection volume was 50 μ L and the run time was 30min. Retention times were 24min and 9min for DXP and DXM, respectively, 21min for TestD (IS of DXP) and 26min for DXA (IS of DXM). Calibration curves of DXP and DXM were linear, respectively in the range 0.5-100 μ g/mL ($R^2=0.9997$, $y=0.2199x-0.0165$) and 0.1-100 μ g/mL ($R^2=0.9974$, $y=1.056x+0.1445$).

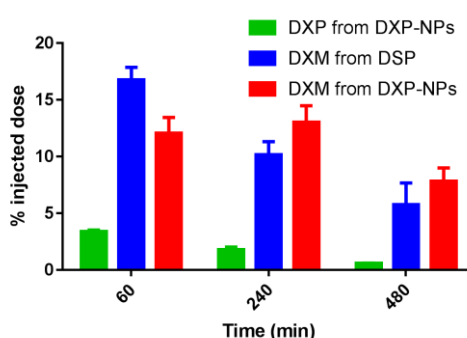


Fig. S7: Biodistribution in liver of DXP-NPs (DXP in green, DXM in red) and DSP solution (DXM in blue) after 12mg/kg (eq.DXM) injected dose on male DBA/1 mice. Results are presented as mean \pm SEM (n=5).