Supplementary Material and Methods

Preparation of Rituximab engineered nanoparticles

Rituximab engineered NP (NP-Rt) were prepared starting from unloaded (NP) PLGA-nanoparticles applying the methodology for Ab-surface engineering of NP. Briefly, in the presence of 150 mg of 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Sigma Aldrich, Saint Luis, MO) and 50 mg of N-Hydroxy-succinimide (NHS, Sigma Aldrich), used to conjugate the free primary amine groups on the particle surface with the carboxylic groups on the antibody molecules, 50 mg of each NP preparation was suspended and stirred at room temperature for 1 hour in 2-(N-morpholino) ethanesulfonic acid (MES, Sigma Aldrich) buffer. The activated NP were collected by ultracentrifugation at 15.000 rpm for 10 min at 4°C and the excess of reagents were removed. NP were re-suspended in MES and stirred at room temperature for 1 hour with the designated volume of Rituximab (100 µl of 1 mg/mL stock solution) in order to obtain Rituximab engineered NP (NP-Rt). After the reaction, the suspension of the engineered NP-Rt were collected by centrifugation and further washed twice by distilled water. NP-Rt were then lyophilized (LyoLab 3000, Heto-Holten, Allerod, Denmark) using D-(+)-trehalose (Fluka-Sigma Aldrich) as cryo-preservative at 1:3 w/w polymer/trehalose ratio.

Physicochemical characterization of the nanoparticles

Mean particle size and polydispersivity index (PDI) of the NP (in distilled water) were determined at 25°C by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern, UK; Laser 4 mW He-Ne, 633 nm, Laser attenuator Automatic, transmission 100-0.0003%, Detector Avalanche photodiode, Q.E.> 50% at 633 nm, T = 25°C). The results were normalized with respect to a polystirene standard solution. The *z*-potential was measured (in similar–plasma fluid, pH 7.4±0.1) using the same equipment with a combination of laser

doppler velocimetry (LDV) and phase analysis light scattering (PALS). All the data are expressed as means of at least three determinations carried out for each preparation lot (three lots for each sample). As the PVA associated with the particles could affect the physical properties and the cellular uptake, the residual PVA was determined by a colorimetric method based on the formation of the colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule (**1**). Briefly, 5 mg of a lyophilized sample were solubilized in 1 mL of DCM. Then, 2 mL of distilled water were added and the organic solvent was evaporated at room temperature under stirring (2 hours). The suspension was filtered (cellulose nitrate filter, porosity 0.45 μm, Sartorius, Firenze, Italy) to remove the polymeric residue and 1 mL of the aqueous solution was treated with 2 mL of 0.5 M NaOH for 15 min at 60 °C. The solution was neutralized with 900 μL of 1 NHCl and the volume adjusted to 5 mL with distilled water. Then, 3 mL of a 0.65 M solution of boric acid, 0.5 mL of a solution of I2/KI (0.05 M/0.15 M), and 1.5 mL of distilled water were added. PVA concentration was determined measuring the absorbance at 690 nm after 15 min incubation at room temperature in comparison with a standard plot of PVA prepared under the same experimental conditions.

The morphology and the surface structure of the NP were assessed by atomic force microscopical observations performed with an atomic force microscope (Park Instruments, Sunnyvale, CA). The measurements were obtained at about 20°C operating in air and in non-contact mode using a commercial silicon tip-cantilever (high resolution noncontact "GOLDEN" Silicon Cantilevers NSG-11, NT-MDT, tip diameter of 5-10 nm; Zelenograd, Moscow, Russia) with stiffness about 40 Nm^{-1} and a resonance frequency around 160 kHz. After the purification and after freeze-drying processes, the sample was dispersed in distilled water before to be applied on a freshly cleaved mica disk (1 cm×1 cm). Two min after the deposition, the water excess was removed using blotting paper. The AFM images were obtained with a scan rate 1 Hz and processed using a ProScan Data Acquisition software.

Analysis of the presence and functionality of Rituximab on NP-Rt.

The presence of Rituximab molecules on the surface of NP-Rt was first indicated by electron spectroscopy for chemical analysis (ESCA), showing the presence of nitrogen atoms on the surface of antibody-engineered NP, as previously described (**2**). ESCA was performed on a XRC 1000 X-ray source analysis system (Specs Surface Nano Analysis, Germany) and an Phoibos 150 hemispherical electron analyzer (Specs Surface Nano Analysis, Germany), using MgKα1,2 radiations. The spectra were recorded in fixed retardation ratio (FAT) mode with 40 eV pass energy. The pressure in the sample analysis chamber was ca. 10-9 mbar. The data were acquired and processed using the SpecsLab2 software. The presence of functional Rituximab on the surface of the NP-Rt, was then investigated by assessing: *i*) the binding to CD20 antigen on the cellular surface; *ii*) the ability to trigger complement activation (as reported in the **Material and Method** section on the main document and below). Briefly, for the binding to CD20 antigen, NP and NP-Rt containing a fluorescent dye were prepared using the same procedure described above substituting 3 mg of PLGA RG 503H with 3 mg of PLGA RG 502H conjugated with Rhodamine B piperazine amide (RBP; Sigma-Aldrich, St. Louis, MO), as previously described (**3**). $CD20^+$ (JMV-2) and $CD20^-$ (OCI) leukemic cells (American Type Culture Collection, Manassas, VA) were incubated with either Rhodamine-labeled NP or Rhodamine-labeled NP-Rt for 1 hour, at 4°C (to inhibit endocytosis). After washing with cold PBS, to remove the unbound NP/NP-Rt, cells were analyzed by flow-cytometry (FACScan, Becton Dickinson, San Jose, CA).

Visualization of Rituximab content in the NP-Rt preparations was carried out by electrophoresis separation on polyacrylamide gel and visualization by silver staining method (Sigma-Aldrich), used because of its high sensitivity. For quantification of Rituximab in the NP-Rt preparation, 100 μg of NP-Rt or NP (loaded as negative control) were run in the same gel together with scalar doses of free Rituximab (range: 200-2 ng), used to set a standard curve

after estimation of the densitometry values by the ImageQuant TL software (GE Healthcare, Buckinghamshire, UK).

Analysis of complement activation

For complement activation assay, JVM-2 cells ($5x10^5$/mL) were incubated with the different NP preparations, or with Rituximab, used as positive control, for 1 hour at room temperature, prior to addition of pooled normal AB human serum (HS, 25%) (Sigma Aldrich), used as a source of complement. Complement activation was evaluated early after addition of HS (30 minutes) by C3 and C4 fragment deposition assessed by flow cytometry, after incubation of cells with goat anti-human C3 or goat anti-human C4 Ab (The Binding Site, Birmingham, UK) followed by FITC-conjugated anti goat Ab (Dako, Glostrup, Denmark).

Evaluation of Nutlin-3 encapsulation efficiency, drug content and drug entrapment efficiency

The drug loading into the NP and NP-Rt was determined by a direct methodology and by using the reverse phase isocratic mode of high performance liquid chromatography (RP-HPLC) method. The HPLC apparatus (JASCO Corp., Tokyo, Japan) comprised a Model PU2089 pump provided of an injection valve with a sample 50 µL loop (Model 7755-023, Rheodyne, Cotati, CA) and a Model 1565 variable wavelength UV/visible detector. Chromatographic separation was carried out on a 10 cm X 4 mm, 3 µm particle size ChromoSep C18 (Varian, Palo Alto, CA). Briefly, 5 mg of freeze-dried Nutlin-3-loaded nanoparticles (either NP-Nut or NP-Rt-Nut) were dissolved in 1 mL of dichloromethane (DCM, Sigma-Aldrich). Then, 5 mL of ethanol were added, since the diffusion of ethanol in DCM leads the precipitation of polymer. The mixture was filtered (0.2 µm porous filter, Sartorius, Firenze, Italy) to remove the polymer residue and the collected solution was analyzed for Nutlin-3 concentration by RP-HPLC using a mobile phase of 0.02 M potassium dihydrogen

phosphate, acetonitrile and methanol in the ratio 45:35:20 (v/v/v), at a flow rate of 1 mL min^{-1}, at 30°C with thermostat. The Nutlin-3 peak was measured at wavelength of 260 nm and quantitatively determined by comparing with a standard plot. Triplicate samples (supernatants) were analyzed. The drug loading was expressed as mg of Nutlin-3 encapsulated into 100 mg of formulation (encapsulated drug+polymer), while encapsulation efficiency was calculated as the percentage of encapsulated drug related to the initial amount of drug used in the preparation.

Analysis of nanoparticle-cellular uptake

For transmission electron microscopy, cells were seeded at $1x10^6$/mL, treated with NP and incubation at 37°C. Cells were then centrifuged at 2000x g for 5 min and the pellet fixed with 2.5% glutaraldehyde/0.1 M PBS (pH 7.3) overnight at 4°C. After several rinses with PBS, the cells were post-fixed in 1% osmium tetroxide at 4°C for 3 hours and then rinsed thoroughly with distilled water, dehydrated by graded ethanol, and freeze-dried. The specimens were sputter-coated with platinum and observed with a transmission electron microscope (TEM, H-800; Hitachi, Tokyo, Japan).

For fluorescence microscopy, cells were seeded at $1x10^6$/ml and exposed to Rhodamine labeled NP and incubated at 37°C in the dark. Cells were washed three times with cold PBS to eliminate extracellular NP before analysis of cellular fluorescence by using an Axiophot fluorescence microscope (Zeiss, Oberlochen, Germany).

B-CLL mouse xenograft models

Female SCID mice (5 weeks-old) were purchased from Charles River Laboratories (Hollister, CA) and were maintained in accordance with the guide for the care and use of laboratory animals at the animal facility of the University of Ferrara. Mice were housed in vented cabinet with food and water ad libitum. The procedures involving animals and their care were approved by the institutional animal ethical care committee (CEASA) of the University of Ferrara and by the Italian

Ministry of Health. JVM-2 (10^7) cells were harvested, washed and suspended in PBS before subcutaneous injection (in a volume of 100 μL) into the right dorsum of 6-week-old mice. When tumors reached 50 mm^3 of volume, leukemia xenograft mice were randomized into groups (of at least 10 mice each) receiving every other day for a total of three times subcutaneous intra-tumoral injections (in 100 μL PBS) of: free native Nutlin-3 (used at 65 μg/mouse); NP and NP-Rt (both used at 1 mg NP/mouse); NP-Nut and NP-Rt-Nut (both used at 1mg NP/65 μg Nutlin/mouse). Additional controls were represented by mice injected with vehicle (PBS) alone. Animals were monitored daily for changes in weight, side effects or signs of sickness. Tumor growth was determined by caliper measurements of two orthogonal axes and the tumor volume was calculated by the formula: $L \times l^2 \times 0.5$, wherein l is the shorter and L is the longer axis; the tumor density was assumed to be equal to one. Survival was calculated as the duration of the animal life span from the inoculation of first treatment until death. Animals were sacrificed when excessive signs of sickness were observed. Necropsy was carried out to determine macroscopic extent and histological characteristics of the subcutaneous masses. In addition, major organs were harvested for microscopic examination and to evaluate the pattern of dissemination of the engraftment.

Histopathological and immunophenotypical analysis

Animal specimens were fixed in 10% buffered-formalin solution and embedded in paraffin. For morphological analysis, 5-μm-thick sections were cut from paraffin blocks and stained with hematoxylin-eosin. Immunohistochemistry was performed by using the primary Ab for human CD20 (clone L26) (Novocastra™; Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK), associated to the NovoLink™ Polymer Detection System (Novocastra) that was used for the visualization of the primary Ab, following manufacturer's instruction. After staining, the slides were examined under a Leica DM2000 optical microscope and microphotographs were taken using a Leica DFC320 digital camera. CD20 surface expression of JVM-2 leukemia cells was analyzed by flow cytometry using PE-conjugated anti-human-CD20 monoclonal Ab (Becton Dickinson). Non-

specific fluorescence was assessed by incubation of cells with isotype-matched conjugated Ab (Becton Dickinson).

Statistical analysis

Results from at least three independent experiments are reported as the means±SD and analyzed for statistical significance by the two-tail Student's t-test and Mann-Witney rank-sum test. Analysis of survival data was carried out with GraphPad Prism version 5 (GraphPad Software); in particular differences in survival between treatment groups were calculated using the Kaplan-Meier curve and survival distribution of the treated and control groups was compared using the Log-rank and the Gehan-Breslow-Wilcoxon tests. Differences were considered significant when p value was <0.05.

Supplementary References

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