Nanoparticles engineered with Rituximab and loaded with Nutlin-3 show promising therapeutic activity in B leukemic xenografts

Running title: Rituximab plus Nutlin-3 loaded nanoparticles

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Statement of translational relevance: Nutlin-3 has shown \textit{in vitro} anti-cancer activity in a variety of p53\textsuperscript{wild-type} cancers, but its potential clinical use is limited by some lacunae with respect to solubility, accessibility to tumor tissues and nonspecific targeting. Therefore, the authors have assessed the anti-leukemic activity of Nutlin-3 encapsulated into poly(lactide-co-glycolide) nanoparticles (NP-Nut) and into Rituximab (anti-CD20 antibody)-engineered NP (NP-Rt-Nut) and of nanoparticles engineered with Rituximab alone (NP-Rt). When tested \textit{in vitro} in the p53\textsuperscript{wild-type} JVM-2 B-leukemic cells, NP-Nut and NP-Rt-Nut exhibited a comparable ability to activate the p53 pathway. In addition, NP-Rt-Nut, as well as NP-Rt, promoted the activation of the complement cascade. When assessed \textit{in vivo} in a JVM-2 xenograft SCID mice model, NP-Rt-Nut promoted a significantly higher survival rate with respect to NP-Nut and to NP-Rt. The results of this study provide insight for further clinical evaluation of NP-Rt-Nut in p53\textsuperscript{wild-type} B cell malignancies, including p53\textsuperscript{wild-type} B-chronic lymphocytic leukemia.
Abstract

**Purpose:** Since the non-genotoxic inhibitor of the p53/MDM2 interactions Nutlin-3 has shown promising *in vitro* therapeutic activity against a variety of p53\textsuperscript{wild-type} cancer cells, in this study we evaluated an innovative strategy able to specifically target Nutlin-3 towards CD20\textsuperscript{+} malignant cells.

**Experimental Design:** The cytotoxic effects of Nutlin-3 encapsulated into poly(lactide-co-glycolide) nanoparticles (NP-Nut) and into Rituximab (anti-CD20 Antibody)-engineered NP (NP-Rt-Nut) as well as of nanoparticles engineered with Rituximab alone (NP-Rt) were initially analyzed *in vitro* in JVM-2 B-leukemic cells, by assessing both the functional activation of the p53 pathway (by Nutlin-3) and/or the activation of the complement cascade (by Rituximab). Moreover, the potential therapeutic efficacy of NP-Nut, NP-Rt and NP-Rt-Nut were comparatively assessed *in vivo* in CD20\textsuperscript{+} JVM-2 leukemic xenograft SCID mice.

**Results:** Functional *in vitro* assays demonstrated that NP-Nut and NP-Rt-Nut exhibited a comparable ability to activate the p53 pathway in the p53\textsuperscript{wild-type} JVM-2 leukemic cells. On the other hand, NP-Rt and NP-Rt-Nut, but not NP nor NP-Nut, were able to promote activation of the complement cascade. Of note, the *in vivo* intra-tumoral injection in JVM-2 B leukemic/xenograft mice demonstrated that NP-Rt-Nut displayed the maximal therapeutic activity promoting a survival rate significantly higher not only with respect to control animals, treated either with vehicle or with empty NP, but also with respect to animals treated with NP-Nut or NP-Rt.

**Conclusions:** Our data demonstrate for the first time the potential anti-leukemic activity of Rituximab-engineered Nutlin-3 loaded NP in xenograft SCID mice.
Introduction

The non-genotoxic activator of the p53 pathway Nutlin-3 induces the rapid accumulation of p53 protein and the consequent induction of cell cycle arrest, senescence and apoptosis in a cell type specific manner (1). Previous studies have shown that Nutlin-3 has little toxicity in animal models, suggesting that the selective non-genotoxic p53 activation by Nutlin-3 might represent an alternative to the current cytotoxic chemotherapy for a variety of p53\textsuperscript{wild-type} cancers, including hematological malignancies (2, 3). The attractiveness of Nutlin-3 with respect to conventional chemotherapy is represented by the lower risk of Nutlin-3 to induce drug resistance and the absence of genotoxic damage. In this respect, it is noteworthy that Nutlin-3 is currently under phase I clinical trial as oral formulation, RO5045337 (protocol ID: NCT00623870), in several Centers located in Canada, USA, UK and Italy. This study will determine the maximum tolerated dose of RO5045337 and the optimal associated 4 weekly dosing schedule of RO5045337, administered as monotherapy in patients with hematologic neoplasms.

Nutlin-3 is a cis-imidazoline compound soluble in organic solvents such as ethanol, DMSO and dimethyl formamide, while it is sparingly soluble in aqueous buffers. So far, administration of Nutlin-3 in mouse models of disease has been performed mostly per os, with a huge amount of drugs required (200-400 mg/Kg twice a day) and some difficulties in giving the correct amount to each mouse (1, 4-7). In order to improve the delivery and efficacy of this drug for the treatment of solid tumors, recent studies have used Nutlin-3 encapsulated into poly(lactide-co-glycolide) (PLGA) nanoparticles (NP) (8, 9), being PLGA the most utilized polymers for the preparation of drug delivery systems (10, 11).

Standard treatments for B cell malignancies, such as B-chronic lymphocytic leukemia (B-CLL), the most common lymphoid malignancy in western countries, include mono- or poly-chemotherapies, usually combined with monoclonal antibodies (Ab), such as anti-CD20 Rituximab (12). In this respect, a recent meta-analysis showed that patients affected by B-CLL receiving
chemotherapy plus Rituximab benefit in terms of overall survival (OS) as well as progression-free survival (PFS) compared to those with chemotherapy alone, especially fludarabine or fludarabine and cyclophosphamide (R-FC regimen) (13).

On these bases, the aim of our study was to assess in vitro and in vivo the use of Nutlin-3 encapsulated in PLGA NP (NP-Nut) and in Rituximab-engineered NP (NP-Rt-Nut), with the hope to propose an innovative strategy able to combine the therapeutic efficacy of Nutlin-3 with the specific targeting towards CD20+ malignant cells of Rituximab. For this purpose, we have employed the SCID-JVM-2 xenograft as model of p53\textsuperscript{wild-type} B-CLL (14, 15), and more in general of B cell malignancies, taking advantage of the fact that the CD20\textsuperscript{+} JVM-2 cell line has been well characterized for the response to Nutlin-3 and other anti-leukemic molecules (16, 17).
Materials and Methods

Preparation of nanoparticles

Nutlin-3 was purchased from Cayman Chemical (Ann Arbor, MI). Nutlin-3 loaded PLGA-nanoparticles (NP-Nut) were formulated by oil-in-water single emulsion–solvent evaporation technique with slight modifications (18, 19). In brief, a solution of 100 mg PLGA RG 503H (Boehringer-Ingelheim, Ingelheim am Rhein, Germany) and 10 mg Nutlin-3 (10% w/w dry weight of polymer) in 3 mL of chloroform was emulsified in 12 mL of 1% w/v aqueous solution of polyvinyl alcohol (PVA; degree of hydrolysisation 86-89 Mol%, viscosity of the 4% w/w water solution at 20°C 3 mPas; Fluka, Deisenhofen, Germany) to form an oil-in-water emulsion (19). Rituximab (anti-human CD20 Ab-Mabthera; Roche, Basel, Switzerland) engineered NP were prepared starting from unloaded (NP-Rt) or from Nutlin-3 loaded (NP-Rt-Nut) PLGA-nanoparticles applying the methodology for Ab-surface engineering of NP previously described (19-21). Further details on NP-Rt preparation as well as on NP and NP-Rt physicochemical characterization are reported into the Supplementary Material and Methods section.

Cell cultures and cell treatments

JVM-2 human leukemic cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI-1640 (Lonza, Walkersville, MD) containing 15% FBS (Gibco BRL, Gaithersburg, MD), glucose 4.5 g/L, sodium pyruvate 1 mM, sodium bicarbonate 1.5 g/L, HEPES 10 mM (all from Gibco). For the different functional assays described in the subsequent paragraphs, cells were seeded at the concentration of 10^6 cells/mL before treatment with NP, NP-Nut, NP-Rt and NP-Rt-Nut, all used at a concentration in the range of 0.06-0.07 mg of NP/mL and normalized for the drug content. As positive controls, and for comparison, cells were also treated with either free Nutlin-3 (Cayman Chemical) or free Rituximab (anti-human CD20 Ab-Mabthera; Roche), based on the functional assay.
Analysis of p53 pathway

Analysis of p53 activation was performed by Western blot analysis. For this purpose, JVM-2 cells were treated with different NP preparations, or with free Nutlin-3, used as positive control, for 24 hours and lysed in ice-cold Ripa buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate) supplemented with protease inhibitors (Roche, Basel, Switzerland) on ice for 1 hour, as previously described (22). Protein determination was performed by using the BCA protein assay (Thermo Scientific, Rockford, IL). Equal amounts of protein for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters (23). Hybridizations were performed by using anti-p53 (DO-1), anti-MDM2 (SMP14), anti-p21 (C-19) and anti-tubulin monoclonal Ab (all from Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with peroxidase-conjugated anti mouse or anti rabbit IgG (Sigma-Aldrich), specific reactions were revealed with the ECL Lightning detection kit (Perkin Elmer, Waltham, MA).

Analysis of cell cytotoxicity

At 24-48 hours post-treatment with the different NP preparations, or with free Nutlin-3 used as positive control, cells were analyzed for total cell viability evaluated by Trypan blue dye exclusion, as previously described (24). In parallel, the degree of apoptosis was determined by Annexin V/7-amino-actinomycin D (7-AAD) double staining (BD Biosciences Pharmingen, Franklin Lakes, NJ) and flow cytometry analysis (FACScan, Becton Dickinson, San Jose, CA), as previously described (25, 26). For analysis of cell cycle profile, cells were incubated with 50 µM 5-bromodeoxyuridine (BrdU; Sigma Aldrich) at 37°C for 1 hour, then anti-BrdU Ab (BD Biosciences Pharmingen, Franklin Lakes, NJ) was bound to BrdU and the complex was detected by FITC-conjugated secondary Ab (Beckman-Coulter, Marseille, France). Cells were stained with 50 µg/mL propidium iodide (PI; Sigma-Aldrich) and analyzed by flow cytometry, as previously described (27).
For complement-dependent cytotoxicity (CDC) assay, experiments were performed as previously described (28, 29) with some modifications. After cell exposure to the different NP preparations and after the addition of pooled normal AB HS (25%), cultures were incubated at 37°C for 24-48 hours before determination of the percentage of induced dead cells.

Comprehensive description of Materials and Methods used for Analysis of complement activation, B-CLL mouse xenograft models, Histopatological and immunophenotypical analysis, Statistical analysis, are reported into the Supplementary Material and Methods section.
Results

Physicochemical characterization of NP-Nut, NP-Rt and NP-Rt-Nut

The composition and the main physicochemical characteristics of the different NP preparations are reported in Supplementary Table 1. Of note, the Nutlin-3 content in 100 mg of NP formulation ranged from 5.0±0.7 mg (encapsulation efficiency, EE of 59±7%) for NP-Nut, to 4.0±0.8 mg (EE of 44±8%) for Rituximab engineered NP (NP-Rt-Nut). This difference, although not significant, could be the consequence of the diffusion of Nutlin-3, probably from the outer portion of the NP, during the conjugation of the antibody and the following purification procedures.

In order to support the presence of Rituximab on NP surface, beside the conventional method of analysis of the diameter size based on photon correlation spectroscopy, NP preparations were analyzed by atomic force microscopy (AFM) (Figure 1A). With this method, surface alterations after conjugation with Rituximab arise from the comparison of images taken on antibody-free and antibody-engineered NP. Indeed, the topographical AFM images showed the antibody-free NP (NP) formed by separated elements and Rituximab-engineered NP (NP-Rt) characterized by larger aggregates of irregular shape (Figure 1A). Moreover, the surface of NP-Rt appeared rough and heterogeneous with presence of gaps and more complex areas (as indicated by the arrows in Figure 1A). Analysis of the height of NP in the 3D images showed that, accordingly with the size measured by PCS analysis, the diameters of antibody-free NP were in the range of 150–200 nm, corresponding to elements with spherical shape. On the other hand, NP-Rt were characterized by an irregular contour and a diameter distributed into a wider range (100-300 nm).

The coupling of Rituximab on the NP surface was indirectly suggested also by electron spectroscopy for chemical analysis (ESCA), performed to measure the elemental composition of the NP (Figure 1B). Analyzing the atom spectra of antibody-engineered NP (NP-Rt) in comparison with antibody-free nanoparticles (NP) it emerged that the spectra obtained after the conjugation with Rituximab showed N signal (for nitrogen atoms) suggesting the presence of the antibody on
NP surface, with coverage of about 7.4±0.6% (percentage of derivatization). ESCA analysis was carried out also on NP obtained stopping the derivatization after the functionalization with NHS and EDC (that contain atoms of N), just before the addition of Rituximab. Only non-quantifiable traces of N were observed in these samples, and the spectrum of the derivatized NP was superimposable with that of antibody-free NP (data not shown), thus demonstrating that the contribute of N in NP-Rt spectrum was really due to the presence of Rituximab on NP surface.

The presence of functional Rituximab on the surface of the NP-Rt was definitively documented by assessing the binding to CD20 antigen on the cellular surface. As shown in Figure 1C, only NP-Rt, but not antibody-free NP, were able to bind specifically the cellular surface of CD20+ (JMV-2) leukemic cells, while not significant binding was detected on CD20- (OCI) leukemic cells. Moreover, analysis by SDS-PAGE and silver staining (Figure 1D) indicated a content of Rituximab on the NP-Rt estimated of approximately 80±10 ng of antibody/100 µg of NP (corresponding to approximately 3.089 femtomoles of antibody/NP). In parallel, the cellular internalization of PLGA NP by endocytosis (30, 31) was confirmed by microscopic examination upon exposure of cells to NP for 24 hours at 37°C, followed by intracellular analyses (Supplementary Figure 1A-B).

**In vitro assessment of p53 and complement activation by the different NP preparations**

In order to ascertain the functionality of the different NP preparations, NP-Nut, NP-Rt and NP-Rt-Nut were analyzed for their ability to activate the p53 pathway in vitro using the p53 wild-type JVM-2 leukemic cell line, as a model system of B cell neoplasms. As shown in Figure 2A, NP-Nut and NP-Rt-Nut showed a comparable ability to promote the accumulation of p53 protein and to induce the expression of p53 specific targets, MDM2 and p21, thus confirming that NP preparations contained functional Nutlin-3. Moreover, NP-Nut and free Nutlin-3 exhibited a comparable ability in terms of p53 induction, while no effect on p53 was observed upon exposure to empty control NP (Supplementary Figure 2).
In parallel, the same NP preparations were tested for their capacity to trigger the complement cascade in the CD20^+ JVM-2 cells. In the presence of human serum, while the antibody free-NP (and NP-Nut) were unable to promote C3 fragment deposition (Figure 2B), NP-Rt clearly induced the deposition on the cell surface of the C3 fragment (Figure 2B). NP-Rt-Nut showed a profile of complement deposition indistinguishable from that of NP-Rt alone (data not shown). These data were remarkable since indicated that, in spite of being engineered on the NP surface, Rituximab was still able to trigger complement activation. In order to distinguish between classical or alternative complement activation pathways, we next analyzed whether NP-Rt were able to trigger C4 fragment deposition. The validation of the classical pathway of complement activation by NP-Rt (and NP-Rt-Nut), but not by NP, came from the analysis of C4 fragment deposition, selectively induced by exposure to NP engineered with Rituximab (Figure 2B).

**In vitro anti-leukemic cytotoxic effects of the different NP preparations**

The biological effects mediated by Nutlin-3 encapsulated into NP were next evaluated on JVM-2 cell cultures, by analysis of cell viability (Figure 3A), apoptosis (Figure 3B), and cell cycle (Figure 3C). The preparations loaded with Nutlin-3 (NP-Nut and NP-Rt-Nut) were able to induce cytotoxic events resulting in reduction of cell viability >60% (Figure 3A), arising from the combination of apoptosis induction and cell cycle block (Figure 3B-C). The results were quantitatively and qualitatively comparable for both NP-Nut and NP-Rt-Nut preparations, while NP alone and NP-Rt showed no significant cytotoxic effects with respect to untreated cultures (Figure 3A-C). No interference due to the antibody presence was observed. Since it is known in literature that the anti-CD20 Ab Rituximab does not exhibit significant cytotoxic effect on CD20^+ B-cells when used *in vitro* under standard culture conditions (32), cytotoxicity of Rituximab-engineered NP (NP-Rt and NP-Rt-Nut) was next evaluated in complement-mediated cell death (CDC) assays, performed using normal human serum (25%) as source of complement (Figure 3D). These experiments clearly indicated that Rituximab exposed on NP surface (NP-Rt), by activating the
complement cascade, was able to trigger >40% cell killing (Figure 3D). Similar cell killing activity was observed upon exposure to NP-Rt-Nut (Figure 3D).

**Treatment with NP-Rt-Nut prolonged the survival of JVM-2 xenografts more efficiently than NP-Nut and/or NP-Rt**

For the *in vivo* evaluation of the therapeutic potential of NP-Nut, NP-Rt and NP-Rt-Nut, we adopted a xenograft model generated in SCID mice upon s.c. injection with a pre-determined optimal number \((10^7)\) of CD20\(^+\) JVM-2 leukemic cells (Figure 4A). JVM-2 xenografts were characterized by subcutaneous tumors, which started to become palpable and measurable by external observation approximately two weeks after cell injections and steadily progressed until mice death with a median survival of 38 days after cell injection. Hystopathological examination of the subcutaneous masses showed that tumors had a solid pattern of growth of CD20\(^+\) positive cells (Figure 4B), with rare infiltration of CD20\(^+\) positive cells in the liver while other organs, such as kidneys and spleen, were unaffected (data not shown). We were first interested in determining the anti-leukemic effects of NP-Nut with respect to free Nutlin-3. Therefore, when tumors reached 50 mm\(^3\), JVM-2 xenograft mice were treated with empty NP, NP-Nut, free Nutlin-3 or control vehicle. No differences in mean survival were observed between NP- and vehicle-mice (as documented in Supplementary Figure 3), which are therefore reported in Figure 4C as a single survival line “Controls” for better clarity. Of interest, treatment with NP-Nut promoted a significant (p<0.05) increase in survival as compared not only to control xenografts, but also with respect to mice treated with free Nutlin-3 (Figure 4C and Table 1). On the other hand, free Nutlin-3 was unable to significantly improve survival with respect to the controls (Figure 4C and Table 1).

Having established that NP-Nut exhibit anti-leukemic activity *in vivo*, we next investigated whether NP functionalized with Rituximab might increase the therapeutic efficacy of the NP loaded with Nutlin-3. In this second round of experiments, we observed that the anti-leukemic efficiency of NP-Rt in our xenograft model was comparable to that reported for NP-Nut in terms of mean
survival (Table 1). Strikingly, treatment of JVM-2 xenografts with NP-Rt-Nut slowed down tumor growth kinetics (Figure 4D) and further enhanced overall survival, with a significant (p<0.05) increase in comparison to the treatments with either NP-Rt (Figure 4E and Table 1) or NP-Nut (Table 1).


Discussion

In the last years, the studies performed by our group have been aimed to investigate the potential therapeutic effects of the innovative non-genotoxic activator of the p53 pathway Nutlin-3 for the treatment of hematological malignancies, including p53\textsuperscript{wild-type} B-CLL (2, 3). The studies performed so far by our and other groups of investigators have strongly suggested that the preclinical evaluation of Nutlin-3 appears highly warranted (2, 3). Interestingly, to overcome the potential problems related to the poor solubility of Nutlin-3, it has been proposed the use of PLGA NP, which showed improved delivery and efficacy of Nutlin-3 \textit{in vitro} in solid tumor cell models (8, 9). However, to the best of our knowledge, NP loaded with Nutlin-3 have never been examined in animal models before the present study.

The use of polymeric NP for drug delivery offers several obvious advantages, such as the possibility to deliver in the target site a great number of active molecules for each unit due to the drug-loading and to the capacity of protecting the active molecules against chemical or enzymatic degradation (33). Another important advantage is the lack of systemic toxicity associated with the use of PLGA for drug delivery (10, 31). Indeed, PLGA is one of the most successfully used biodegradable polymers; its hydrolysis leads to metabolite monomers, lactic acid and glycolic acid, which are endogenous and easily metabolized by the body via the Krebs cycle. PLGA is approved by the US FDA and European Medicine Agency (EMA) in various drug delivery systems in humans (31). Active targeting of NP to tumor cells has been successfully obtained by conjugating ligands to the surface of NPs. Particularly, antibody-engineered nanoparticles have been shown to significantly enhance the efficacy of multiple anticancer drugs in several \textit{in vivo} models of solid tumors (e.g., breast cancer, colon cancer and malignant gliomas) (34, 35). Therefore, in consideration of the wide experience in using Rituximab in the therapeutic approaches for the treatment of B cell malignancies and in particular for B-CLL (12, 13), and taking into account the well-known properties of of PLGA for drug delivery systems (10), we have planned to engineer...
PLGA NP with Rituximab (NP-Rt). Subsequently, also NP loaded with Nutlin-3 were engineered with Rituximab (NP-Rt-Nut). Each preparative of NP-Nut, NP-Rt and NP-Rt-Nut was chemically and functionally characterized evaluating \textit{in vitro} the ability to activate the p53 pathway, to induce cellular apoptosis and inhibition of cell cycle progression, as well as to trigger complement cascade activation. These \textit{in vitro} functional studies were performed by using the same cell type (CD20$^+$ JVM-2) used for the xenograft transplants in which the \textit{in vivo} analyses of the NP preparations were carried out.

The major findings of our study can be recapitulated as follows. First, the chemical and structural characteristics of all NPs tested in this study made them promising for subsequent \textit{in vivo} applications. In particular, the hydrodynamic diameter around 200 nm is reported as optimal for efficient cellular uptake (36), while $\zeta$-potential close to neutrality can prevent macrophage phagocitosis when carriers are administered \textit{in vivo} (37). Second, by combining the morphological and functional analyses, we documented the conjugation of Rituximab on nanoparticles surface supporting the possibility to obtain targeted carriers able to deliver antineoplastic drugs to B tumor cells. Third, NP loaded with Nutlin-3 exhibited good drug content (at about 5 mg/100 mg of formulation) and encapsulation efficiency (close to 50%). This percentage is lower with respect to that reported by previous authors (8, 9) and is likely consequence of the repeated centrifugation required to purified the samples especially both from PVA and from the reagents used during the coupling with antibody. Finally, NP-Nut and NP-Rt-Nut were able to functionally activate the p53 pathway \textit{in vitro}, as also demonstrated by the induction of cell cycle arrest and of apoptosis in JVM-2 cells. On the other hand, NP-Rt and NP-Rt-Nut, but not antibody-free NP or NP-Nut, were able to activate the classical complement cascade promoting CDC \textit{in vitro}. Of great interest, \textit{in vivo} administration of NP-Rt and/or NP-Nut in JVM-2 xenografts exhibited a comparable therapeutic efficacy (in terms of survival rate), that was significantly higher with respect to control mice. Moreover, the anti-leukemic efficacy of Nutlin-3 encapsulated in NP was further enhanced (in a significant manner) by using the Rituximab-engineered NPs loaded with Nutlin-3 (NP-Rt-Nut), in
keeping with their *in vitro* ability to activate both the p53 pathway and the complement cascade, suggesting the occurrence of mechanisms of cell death summarized in Figure 5.

Since many years, nanotechnology has been recognized as an important potential tool for cancer therapy. An appropriate carrier should be able to protect drug, such as Nutlin-3, from metabolic inactivation, ameliorating the delivery by using parenteral administration. Not only, the carrier should be particularly suitable for tumor targeting. In this context, we could demonstrate for the first time that an approach based on the combination of Rituximab and Nutlin-3 on the same NP was not only feasible but also gave significant advantages in terms of survival of the treated animals, probably due to the better targeting of the B-neoplastic cells and to the combination of the pro-apoptotic activity of Nutlin-3 coupled to the ability of Rituximab to activate the complement-mediated cell death also when engineered on the surface of PLGA NP (Figure 5).

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References


Table 1. *In vivo* effects of Rituximab-engineered NP samples.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Survival (median, days)</th>
<th>P (vs Controls)</th>
<th>P (vs Nutlin-3 or NP-Rt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>15-16.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Nutlin-3</td>
<td>21</td>
<td>0.3650</td>
<td>-</td>
</tr>
<tr>
<td>NP-Nut</td>
<td>27.5</td>
<td>0.0088*</td>
<td>0.047&lt;sup&gt;a&lt;/sup&gt;*</td>
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<tr>
<td>NP-Rt</td>
<td>25</td>
<td>0.0027*</td>
<td></td>
</tr>
<tr>
<td>NP-Rt-Nut</td>
<td>36</td>
<td>0.0008*</td>
<td>0.041&lt;sup&gt;b&lt;/sup&gt;*</td>
</tr>
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P was calculated based on Gehan-Breslow-Wilcoxon test; Rt: Rituximab.*: statistically significant; <sup>a</sup>: compared to Nutlin-3; <sup>b</sup>: compared to NP-Rt.
Figure legends

**Figure 1. Characterization of the Rituximab-conjugated NP.** In A, NP antibody-engineering was visualized by AFM surface analysis of NP before (NP; a1-a4) and after conjugation with Rituximab (NP-Rt; b1-b4). Panels show representative topographical images of NP surfaces (1), their magnifications (2) and the “error signal” mode visualization (4). Panels 3 are 3D elaboration of the images showing the height of NP in the vertical axis. Arrows indicate the changes on the surface of NP after conjugation with Rituximab. In B, ESCA analysis of NP and Rituximab-engineered NP (NP-Rt). Picks corresponding to oxygen (O), nitrogen (N) and carbon (C) species are indicated on the plot representing NP and NP-Rt spectra, and are reported in the table together with the corresponding percentage of derivatization. Representative analyses carried out on two different lots of NP-Rt are shown. In C, flow cytometric analysis documenting the binding of NP-Rt, but not of NP, specifically to the cells surface of CD20⁺ (JVM-2; arrowhead) and not of CD20⁻ (OCI) cells. For these experiments, Rhodamine-labeled NP and NP-Rt were used. Representative graphs are shown. In D, visualization of the Rituximab content in the NP-Rt preparations carried out by electrophoresis separation on polyacrylamide gel and silver staining. Scalar doses of free Rituximab (range: 200-2 ng) were run in the same gel together with NP-Rt and NP (loaded as negative control) and used to set a standard curve after estimation of the densitometry values by the ImageQuant TL software (GE Healthcare, Buckinghamshire, UK). MW: protein molecular weight marker.

**Figure 2. In vitro assessment of p53 and complement activation by the different NP preparations.** Leukemic cells, characterized for having p53<sup>wild-type</sup> (JVM-2), were exposed to the indicated NP preparations (used at the concentration of 70 µg/mL). In A, after 24h of NP-treatments, equal amount of cell lysates were analyzed for p53, MDM2 and p21 protein levels by Western blot. Baseline controls are represented by cultures treated with empty NP. Tubulin staining...
is shown as loading control. Western blot results representative of four independent experiments are shown. In B, activation of complement was evaluated in flow cytometry by analyzing the deposition of human C3 and C4 fragments on the cell surface after exposition of cells to NP in the presence of human serum (25%). Activation of complement upon exposure to free Rituximab (Rt; 2 µg/mL) is also shown as positive control. Experiments were performed in triplicate. Control, untreated cultures. Representative graphs are shown.

Figure 3. In vitro assessment of cell cytotoxicity by the different NP preparations. JVM-2 leukemic cells were treated with the indicated NP preparations for 24-48 hours (used at the concentration of 70 µg/mL). Cells treated with free Nutlin-3 (10 µM) are also shown as positive control. In A, cell viability was analyzed by Trypan blue dye exclusion and is expressed as percentage of the untreated cultures, set to 100% (dashed line). In B, the percentage of apoptotic cells was determined by flow-cytometry after Annexin-V/7AAD staining. Mean cell apoptosis of untreated culture is indicated by the dashed line. In C, the distribution of cells in the different phases of the cell cycle was calculated after BrdU/PI staining and flow cytometric analysis and is expressed as percentage of the total population. In D, complement dependent cell death (CDC) was evaluated after cell treatment in the presence of 25% human serum. Mean cell death of untreated culture is indicated by the dashed line. In A-D, data are reported as means±SD of at least three independent experiments; asterisk indicates p<0.05 compared to control untreated cultures.

Figure 4. Anti-leukemic activity of Rituximab-engineered Nutlin-3 loaded NP assessed in vivo in a B-CLL like xenograft murine model. SCID mice were subcutaneously injected with JVM-2 cells (10^7 cells/mouse). In A, cell surface expression of CD20 on JVM-2 cells evaluated by flow cytometry. The unshadowed histogram represents the background fluorescence obtained from the staining of the cells with isotype-matched control Ab (Irr.), while the shadowed histogram represents the staining of the cells with the anti-CD20 Ab. In B, representative images of the solid
Subcutaneous tumor, characteristic of the JVM2 xenografts, upon macroscopic observation and microscopic analyses of sections stained with hematoxylin and eosin (H&E) and anti-human CD20 staining (original magnification x200). In C, when xenograft tumors reached 50 mm$^3$ of volume, mice were injected with: empty NP and vehicle (control mice; n=20), naked Nutlin-3 (n=10) or Nutlin-3 loaded NP (NP-Nut; n=10). In D-E, when xenograft tumors reached 50 mm$^3$ of volume, mice were injected with: Rituximab-engineered NP either empty (NP-Rt; n=10) or Nutlin-3 loaded (NP-Rt-Nut; n=10). In D, measurement of subcutaneous tumor volumes was performed every 3 days for the first 12 days after the beginning of treatments, since the early death of control mice limited statistics in the subsequent time points. Results are expressed as mean±SD. Asterisk: p<0.05. In C and E, survival is reported as percentage measured from the day of the first treatment.

Figure 5. Schematic representation of the potential mechanisms of cell death triggered by NP-Rt-Nut in B-leukemic cells.
Figure 1

A

NP

a1)  
a2)  
a3)  
a4)

NP-Rt

b1)  
b2)  
b3)  
b4)
Figure 1

Intensity (a.u.)

Binding Energy (eV)

% of Derivatization

O 1s [at %] C 1s N 1s

NP
NP-RI (I/I0)
NP-RI (II/I0)

37.8
35.9
34.5
62.0
62.0
1.1
1.1
1.3
1.3
8.0
8.0

A
Figure 1

CD20^+ cells (JVM-2)

CD20^- cells (OC)

Relative fluorescence

Untreated
NP
NP-Rt

Untreated
NP
NP-Rt

Untreated
NP
NP-Rt
Figure 2

A

JVM-2

<table>
<thead>
<tr>
<th></th>
<th>NP</th>
<th>NP-Rt</th>
<th>NP-Rt-Nut</th>
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<tr>
<td>p53</td>
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<tr>
<td>Tubulin</td>
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</table>

B

Complement activation

C3 fragment deposition

- Control
- NP
- NP-Rt

Relative fluorescence

C4 fragment deposition

- Control
- NP-Rt
- Rt
Figure 3

A

Cell viability (% of untreated)

B

Cell apoptosis (%)

C

Cell cycle (%)

D

25% Human Serum

CDC (%)

NP NP-Nut NP-RI NP-Rt-Nut Nut

NP NP-Nut NP-RI NP-Rt-Nut Nut
Figure 4

A

B

JVM-2

Surface CD20 expression

Relative fluorescence

Irr. mAb

CD20 mAb

H&E

CD20

[Images of tissue sections: H&E and CD20 staining]
Figure 4

C

Percent survival vs. Days post treatment

- Controls
- Nutlin
- NP-Nut

D

Tumor volume (mm³) vs. Days post treatment

- Control
- NP-Rt
- NP-Rt-Nut

E

Percent survival vs. Days post treatment

- Controls
- NP-Rt
- NP-Rt-Nut

p < 0.05, p < 0.01
Clinical Cancer Research

Nanoparticles engineered with Rituximab and loaded with Nutlin-3 show promising therapeutic activity in B leukemic xenografts

Rebecca Voltan, Paola Secchiero, Barbara Ruozzi, et al.

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