The natural killer cell activation compound 815A: solubility, stability, and injectable formulations

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Abstract

Cancer highly impacts the society, being currently one of the leading causes of death worldwide. Immunotherapies have been found efficient in the treatment of this disease, showing great results in many patients with different types of cancer. However, these encompass high investments per treatment due to therapeutic individuality, thus not being readily available as first-line treatment. Natural killer (NK) cells have been gathering attention considering their essential role in cancer immunosurveillance by rapidly inducing tumor cell death without prior sensitization. In recent work conducted in the CQE-BIOMOL research group, a small organic molecule (**815A**) capable of triggering the cytolytic response of NK cells by engaging one activating receptor of these cells, was developed. Despite its efficacy has been demonstrated, solubility and stability issues hamper more compressive studies of its effect. In this work, a water soluble injectable formulation of compound **815A** was developed. This stable formulation was produced by combining the test compound with a surfactant (mannitol), enabling further studying of compound **815A**. This allowed the determination of the *logP* value of the molecule, its plasma protein binding profile, plasma stability and the development of a suitable quantification method. In addition, a controlled-release system was developed to control the activation of NK cells by **815A**. The prolonged NK cell activation effect was achieved with polymeric nanoparticles with biocompatible and biodegradable characteristics that released the test compound at a steady rate, preventing the over-activation and subsequent repression of NK cell response.

Keywords: NK cells; Small organic molecule; Immune response triggering; Formulation; Drug delivery system

1. Introduction

According to the European Commission's database, in 2020 approximately 2.7 million new cases of cancer were reported, along with 1.3 million deaths associated to oncologic conditions, in the member countries of the European Union.[1] Increases in cancer incidence have been reported through the years and, as a consequence of its prominent impact in the society, cancer has been receiving a lot of attention in terms of funding for the discovery of new treatments, ways to minimize the associated side effects and new prevention measurements.[2] Immunotherapy has demonstrated its effectiveness in a large number of patients and several types of cancer, having demonstrated that is able to restore a state of antitumor immunosurveillance.[3] However, there is not a single therapy falling into this category that is effective in every patient and in every single cancer type. Effective treatments would, therefore, possibly require the development of technologies specifically for each person which, in terms of costs, will most likely not be conducted.[3,4] The economical aspect of these kinds of treatments is a major drawback, making immunotherapy inaccessible for the majority of the patients. For all these reasons, investment in new immunotherapies or immunotherapies-alike are required to ensure that they go from being the last source of hope to be the first form of acting against the disease. On that aspect, natural killer cells have been collecting the researchers' attention due to their ability to detect and kill transformed cells, being proposed as the next major target for cancer fighting.[5,6] They are a part of the innate immune system and are known for being able to exert cytotoxic activity without prior sensitization, as well as an immunomodulatory activity through the production and secretion of cytokines.[7] NK cell response is a direct consequence of the integration of both inhibitory and activating signals arising from receptors present on its surface. Most of these receptors are only present in these cells and are constitutively expressed, independent on the activity state of the cells.[8] Evidences have demonstrated that the dominant activating receptor, responsible for the cytolytic activity against several tumor types, is the Natural Killer protein 30 receptor (NKp30).[9] Previous work developed in the laboratories of the Design, synthesis and toxicology of bioactive molecules (BIOMOL) group of Centro de Química Estrutural (CQE) pursued the hypothesis of using small organic molecules to trigger the activity of NK cells through binding of the NKp30 receptor.[10] In this work, computational studies were conducted to examine the structure of the receptor and develop a series of molecules with high affinity towards the binding site. During the development, structural characteristics that could interfere with physicochemical features important for further pharmacological and toxicological studies, namely the molecule's solubility, were considered. This included, among others, the addition of specific chemical moieties, such as a quaternary amine group to increase aqueous solubility. After several modifications of the initial hit molecules, compound **815A** (Figure 1) was identified as a good ligand for NKp30. Extensive biological testing demonstrated that this was able to trigger the cytolytic responses of NK cells *in vitro*. Depending on its concentration, compound **815A** is able to induce different responses from NK cells (Figure 1). However, for each response, there is evidence showing that any **815A** concentration above the optimal ranges, depicted in Figure 1 will result in an activity decay. It is hypothesized that the concomitant stimulation of different NKp30 isoforms may result in a downregulation of the response.[11] These differences justify the dual immunomodulatory activities of the NKp30 receptor and, in fact, depending on the expression level of each isoform, the immunological outcome will differ. Thus, overstimulating the NK cells with the **815A** probably results in a counteracting response to the desired, therefore requiring a thorough study to determine the effective therapeutic window. An evaluation of the compound's **815A** structural characteristics according with the Lipinki's rules, indicated that this will be poorly orally active due to its high molecular weight and due to a high number of hydrogen bond acceptors.[12] Moreover, according to an *in silico* tool (Drug Metabolism and pharmacokinetics Analysis Platform (DruMAP))[13], the compound is also expected to have a high solubility, high affinity for plasma proteins and high renal elimination. Considering these characteristics, in the present work, an IV formulation will be developed to overcome administration issues and to better control the circulating levels of **815A** Besides developing a simple free-form formulation, after the complete characterization and determination of key pharmacokinetic-influencing parameters of compound **815A**, a modified release system will be developed and tested to verify its adequacy as a method of controlling the circulating amount of the test compound.

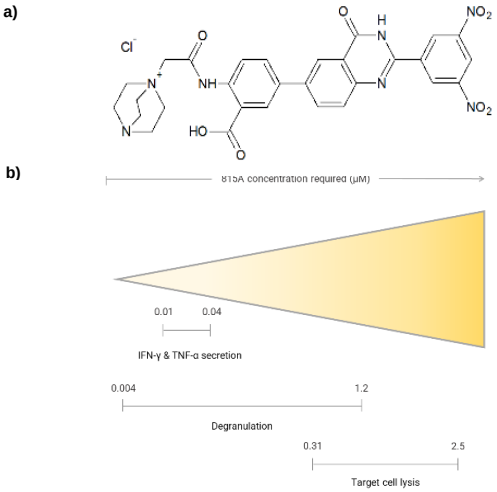

Figure 1: a) Molecular structure of the NK cell activation molecule 815A; b) Concentrations of compound 815A required for inducing different NK cell responses. Schematic representation of the amount of 815A required to induce different NK cell responses, such as cytokine secretion (IFN-γ and TNF-α), degranulation and target cell lysis. Adapted from data retrieved from the original work.[10]

2. Results & Discussion

2.1. Quantification of compound 815A

2.1.1. UV-Vis spectroscopy

The two absorbance maxima of compound **815A** (280 and 330 nm, in DMSO) were found useful for the unequivocal identification of the compound. These same wavelength values were used to plot two calibration curves in DMSO to determine the molar extinction coefficients (Ɛ) of **815A** at both 280 and 330 nm (Figure 3). Both of them present a coefficient of determination (R^2) of approximately 0.999 and, considering the Lambert-Beer's law, the $Ɛ_{280}$ and $Ɛ_{330}$ values found were 24210 and 18100 $M^{-1}.cm^{-1}$, respectively.

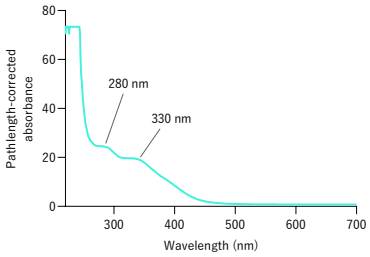

Figure 2: UV-Vis absorption spectrum of compound 815A in DMSO, recorded between 700 and 220 nm. Two characteristic maximum absorbance peaks are observed at 280 and 330 nm.

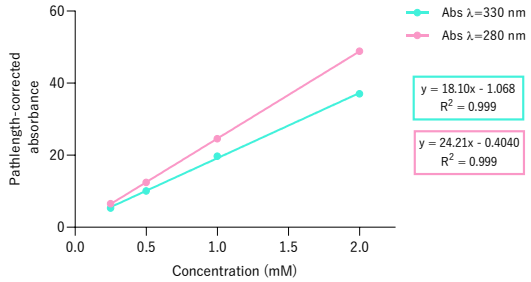

Figure 3: Calibration curves of compound 815A obtained at wavelengths of 280 and 330 nm. The molar absorptivity coefficients were retrieved from the slope of the linear regressions applied to each dataset.

2.1.2. HPLC-UV-Vis

A. Chromatographic separation optimization

Different approaches were attempted to find the better chromatographic conditions to separate and identify compound **815A** in complex matrices. All the results are summarized in Table 1. Tests conducted suggest that the use of HPLC techniques is not indicated for the simultaneous separation and quantification of the test compound. Matrix interferences, together with the dual hydrophobic and hydrophilic characteristics of **815A**, hamper the direct use of HPLC, affecting significantly both reproducibility and sensitivity of the method. Due to these setbacks, to allow the quantification of **815A** in biological matrices, namely blood plasma samples, an appropriate sample preparation method had to be developed and combined with HPLC analysis. Therefore, a standard HPLC procedure was defined, based on the results obtained in the several attempts, to unequivocally identify the presence of the test compound in the samples and to determine its concentration.

B. Quantification process

A well-defined, symmetrical, and sharp peak with a reasonable resolution of compound **815A** could only be obtained using a C18 column with an isocratic elution program consisting of a 1:1:1 mixture of 0.1% of aqueous formic acid – acetonitrile with 0.1% formic acid – methanol, at a flow rate of 1 $mL.min^{-1}$. When assessed, these conditions led to high inter-assay (or inter-day) coefficients of variation (%CV), being all above or near the usually acceptable value of 15%, which translates into a poor method of quantification of compound **815A**. These were identified as being the result of the injection system, being thus mitigated with the use of an internal standard (IS). Anthranilic acid, a precursor for the synthesis of **815A**, was considered since this presents a similar UV-Vis absorption profile to the compound in test, absorbing strongly at *ca.* 330 nm. Moreover, this compound is eluted with an Rt of approximately 3.5 min, after **815A** (Rt of 2.9 min). The resolution attained with the C18 column is sufficient to allow the observation of two individualized peaks in a short time run. It was thus possible to develop an optimized HPLC method to quantify the **815A,** which involves the preparation of **815A** samples containing the internal standard at a concentration of 5 mM in DMSO and further monitoring at 330 nm. With this, the lowest concentration of compound **815A** possible to detect was 0.6 mM, whereas the quantification limit was fixed at 1.8 mM.

2.2. Isolation of compound 815A from biological samples

The lack of a robust HPLC method to simultaneously separate and quantify the **815A** present in biological samples prompted the development of a technique to selectively isolate the compound from complex matrices. Considering the high concentration of proteins in plasma, it was decided to start the development of the isolation procedure by reducing the protein content of plasma samples. There are high probabilities that only a small amount of compound will be found in its free form within the systemic circulation due to its probable high affinity for plasma proteins, meaning that the isolation of **815A** from plasma samples is expected to present some difficulties. Several deproteinization techniques were tested (ultrafiltration, protein's precipitation by adding organic solvents, metal ions, by varying the pH, salting out or heat shock) in plasma-like samples, namely samples composed by 40% of bovine serum albumin (BSA), mimicking the natural content of albumin in blood plasma of around 55%.[14] It was found impossible to isolate compound **815A** form mixtures containing BSA as this was sequestering the compound causing its precipitation or removal in the protein content reduction step. The high affinity of compound 815A for BSA was confirmed after HPLC quantification of the test compound in the supernatants of precipitated protein samples spiked with 815A. The results showed that the majority of the compound added to the mixture co-precipitated with the protein since it was not detected in the supernatant (Figure 4). The test compound could be partially recovered after enzyme digestion of the pelleted proteins, confirming that compound 815A has a very high affinity for plasma proteins.

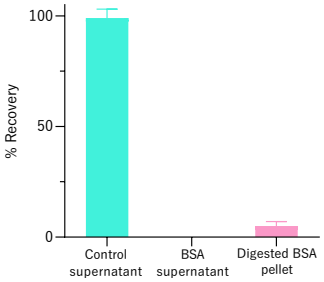

Figure 4: Percentage recovery of compound 815A from samples with and without BSA. Compound **815A** co-precipitates with proteins and it is possible to be, in part, recovered by digesting the pelleted protein. Compound concentrations were determined using the method described in Section 4.2.5. The results are a mean ± SD of three independent experiments.

The only deproteinization technique that allowed the isolation of the compound **815A** from the protein mixture was the addition of a high concentrated solution of a salt. Strikingly, the addition of 5 times the volume of sample of a saturated sodium chloride solution followed by incubation at 3 ºC overnight, resulted not in the precipitation of the proteins but on the selective precipitation of the test compound. The mean recovery percentage of **815A** observed with NaCl was 77% (Figure 5). The effect of adding an additional protease incubation step, using proteinase K, was tested , resulting in only a small increase of the recovery percentage of **815A**.

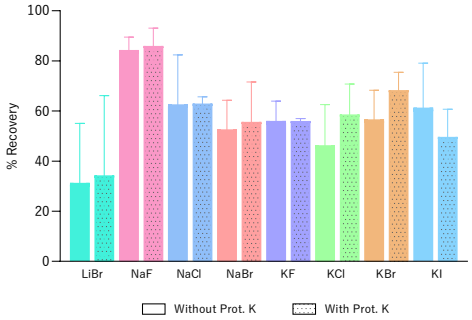

Figure 5: Effect of different salts and of the pre-incubation with proteinase K on the recovery of the test compound from surrogate plasma samples. All assays were performed using saturated solutions of all alkaline metal salts available as precipitating agents. The addition of these salts' solutions to 40% BSA spiked with 0.2 mM **815A** solutions was made with and without the presence of proteinase K (Prot. K). When the enzyme was used, the mixture stayed in incubation at 37 ºC for one hour, and only then the salt solution was added followed by an overnight incubation at 3 ºC. Percentage of recovery assessed using the UV-Vis absorbance at 330 nm. Results are presented as the mean ± SD of three independent experiments.

This suggests that the salt ions are able to release the molecules of **815A** from the proteins and cause their precipitation. However, and although not significant, there are differences in most cases which suggest a beneficial effect of the use of proteinase K in the recovery of the test compound. The major differences, however, are observed when varying the salt used.

Table 1: Summary of the most observed issues during the HPLC quantification process optimization.

Type of chromatography	Stationary phase	Main problems experienced	Possible cause
Reverse Phase	C18	Multiple peaks of the test compound. Impossible to obtain a single isolated peak	Presence of different ionizable groups in the compound structure
		Elution of the compound along and/or before the vehicle	Lack of non-polar interactions between the compound and the stationary phase
	CN	High matrix interference affecting Rt and signal quality	Low sensitivity and reproducibility common for these type of stationary phase
	C8	Elution of the compound along and/or before the vehicle	Lack of interactions between the compound and the stationary phase
Normal Phase	HILIC		
Ion Exchange	C18 – SDS	Non-elution of the test compound from the column	Too many interactions between the compound and the stationary phase
	C18 – Octanoic acid		

While lithium bromide seems to be quite inefficient in promoting the precipitation of compound **815A**, with the use of sodium fluoride a recovery of approximately 85% of the test compound was achieved. The effect here observed seems to be caused by the anion and not the cation, as sodium bromide and chloride had almost the same effect. On the other hand, the use of potassium fluoride resulted in lower recovery percentages when compared to the sodium counterpart. The reason behind this observation is unknow. One possible explanation for this difference might lie on the solubility of these two salts, being the sodium fluoride *ca.* 20 times less soluble than potassium fluoride which may have significant effects on the ionic strength of the mixture and influence the solubility of **815A**. However, the effect of sodium fluoride on the selective precipitation of compound **815A** is quite interesting. Fluoride ions, as well as other anions, are able to establish anion-π interactions that may lead to the formation of stacked structures due to the presence of multiple aromatic rings within the structure of **815A**.[15] For instance, by promoting π-anion-π interactions, in which the π systems are aromatic rings from two different molecules of **815A**, fluoride ions would be contributing to the aggregation of several **815A** molecules causing these to precipitate. In fact, from the four halides tests, fluoride has been found to form the most energetically favorable associations with aromatic systems.[15] Also, the relatively small atomic radius of F, compared to other halides, might contribute to this effect. Although the effect of fluoride in this system could not be explored, it became evident that the use of saturated solutions of sodium fluoride, in combination with pre-incubation of protein samples with proteinase K, resulted in high recovery percentages of compound **815A**. These effects were confirmed in real plasma samples from three different donors, followed by HPLC quantification. A mean recovery of 76% was obtained, which is, in fact, a little bit lower than the previously obtained 86% whilst using the BSA surrogate. This might be due to the higher complexity of the plasma samples that contain components other than proteins that can contribute to maintains the test compound in solution. However, the complexity of the matrix did not influence the selectivity, as the chromatogram of the precipitate showed only the peaks assigned to compound **815A** and the IS.

2.3. Development of an injectable formulation of 815A in free form

At an early stage of development of a New Drug Entity (NDE), an injectable formulation of the compound is a requisite for the pre-clinical phase, namely for *in vivo* pharmacokinetic screening studies.[16,17] Parenteral formulations should be isotonic and euhydric, meaning that their osmolarity and pH should be similar to the physiological values, respectively. However, because most NDEs face solubility and stability problems in aqueous physiological conditions, other conditions are often used. When developing these, one must consider that extreme pH and osmolality values can cause damage on the vascular endothelium and throughout the circulation.[18] Usually the first approaches assessed to increase the compound's solubility is to the adjust the pH of the formulation. In most cases, to be effective, a pH value different enough from the compound's pK_a must be used, while respecting the working range of $3.5 \leq pH \leq 9$ to reduce the risk of local irritation and vein damage. If that's not possible, a slow rate infusion must be considered to overcome those risks. This approach is only suitable for electrolytes, and the compounds that do not fit into this category are frequently mixed with cosolvents (water miscible organic solvents), surfactants and/or complexed with, for instance, cyclodextrins. Sometimes a combination of these techniques is required to completely develop a stable and soluble formulation. On the other hand, some APIs may be administrated using dispersion systems, namely emulsions, liposomes, and others. These, due to their complexity and difficult optimization, are usually the last ones to be attempted.[17-19] At preclinical settings, a combination of pH adjustment and the addition of cosolvents/surfactants is often used to quickly develop an injectable formulation of the test compound.[17] The first logic approach to enhance the compound's solubility would be to adjust the pH of the formulation. Nevertheless, that would require to know all the pK_a values of this compound, in order to buffer the vehicle at the intended pH. However, these could not be determined due to high amount of compound necessary to perform titration experiments. Moreover, this would still require the use of a cosolvent, such as DMSO, to get the compound into aqueous phase and, the effect of different pH values in the compound structure in long term is not known. Therefore, the use of a cosolvent able to increase the **815A**'s solubility was tested. DMSO can be used to prepare concentrated stock solutions of compound **815A** and, from those, aqueous solutions can be prepared. DMSO is classified as a class 3 solvent, meaning that its use up to 0.5% (v/v) encompasses low risks to human health and low toxicity, according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) quality guideline for impurities Q3, namely the Q3C for residual solvents.[20] However, it was decided to avoid the use of this solvent as, in some cases, a yellow precipitate was formed in buffered solutions containing 50 µM of compound **815A** and 0.5% (v/v) of DMSO stored for some time at 4 ºC. In previous work, it was found that compound **815A**, although unable to form stable solutions in water or simple buffers (*e.g.*, phosphate buffer), could remain dissolved in culture medium, with and without serum proteins. This suggests that some of the components of the medium may be acting as surfactants, allowing **815A** to remain solubilized. A surfactant aids the wetting of API particles by preventing their aggregation through electrostatic repulsion or steric hindrance, depending on whether they are ionic or polymeric surfactants, respectively.[19] Hence, considering this, the possibility of developing a formulation constituted by water, **815A** and a surfactant was assessed.

In Table 2 are presented the first tested surfactants, according with the stablished limits for IV formulations within the Inactive Ingredients Database, approved by the Food and Drug Administration (FDA).

Table 2: Surfactants limits for IV formulations according to the Inactive Ingredients Database.[19]

Surfactant	Maximum potency per unit dose
Polysorbate 20	2% (w/v)
Polyvinyl alcohol	2% (w/v)
Albumin	2% (w/v)
Glucose	5% (w/v)

The formulation preparation encompassed the dilution of a stock solution of **815A** in DMSO in the aqueous surfactant solution at a compound/surfactant ratio of 1:20.[19] After a centrifugation step, a yellow precipitate was found in every mixture, with the exception of those using glucose as surfactant. Both polysorbate 20 and polyvinyl alcohol are constituted by large hydrocarbonated chains and have multiple hydroxyl groups. The **815A** precipitation with these two is most probably due to the repulsion between the compound and the hydrophobic chains of the surfactants, reducing the interactions between the two and consequently causing precipitation. Regarding albumin, the precipitation of **815A** was not expected considering the known affinity of **815A** for this protein. However, this might be a consequence of the relatively low amount of protein used in comparison to what is found in circulation and in plasma samples. As for the glucose, it is highly probable that hydrogen bonds are formed with the aqueous medium and with the **815A** leading to its stabilization in solution. In most culture media used in animal cell culture, glucose is added to a final concentration of 1 g/L. It is possible that this relatively high concentration of glucose enabled the stabilization of the compound in solution. Aiming to achieve a practical way to prepare the injectable formulation, glucose/**815A** mixtures were prepared and dried to remove solvents,

including DMSO. The intended result would be a loose powder, easily transferable. Instead, a more jellified pellet, easily dissolved in water, was obtained. Although efficient in solubilizing the test compound and producing a dry formulation easily dissolvable in water, the obtained pellets were difficult to handle and, in some cases, especially when using larger amounts of mixture, their complete drying was difficult. To try to solve this hurdles, other sugars and sugar alcohols were tested, including mannitol, ribose, fructose, inositol, arabinose and sucrose. Every single one of them allowed **815A**'s solubilization and, overall, all dry formulations were dissolvable in water forming clear solutions. However, all dry formulations were obtained as jellified pellets, similar to what was observed with glucose, except the formulations using mannitol or inositol (sugar alcohols). These formed free-flowing powders as it was intended, being the one obtained with mannitol the easiest one to dissolve in water. Considering these results, mannitol was chosen as surfactant for **815A**'s solubilization, and all subsequent assays were conducted using this sugar alcohol. The proportion 1:20 was found to be the one in which was possible to ensure that the obtained solid dispersions were as close to DMSO-free as possible in the final formulation. The use of a high volume of mannitol solution ensured that the proportion water/DMSO was high enough to reduce significantly the boiling point of the mixture[21], reducing drying times and ensuring the complete DMSO removal. The final dry formulation, when subjected to several tests to determine its stability over time and in several experimental conditions allowed the conclusion that the formulation remains stable when stored for long periods of time before and after being redissolved. Moreover, it was also concluded that the full dissolution of the dry formulation cannot be obtained with salt-containing buffers, including 0.9% (m/v) sodium chloride or PBS. However, stable solutions were obtained after the addition of pure water, being then easily dispersed into protein-containing moieties, such as blood plasma, without the formation of any visible precipitates. Finally, the previously developed extraction methodology was tested in plasma samples spiked with the soluble formulation of **815A**. A final mean recovery of 80% confirmed that the selective extraction of compound **815A** is equally effective even when the samples are prepared with the soluble formulation.

2.4. *In vitro* ADME assays

Prior to any *in vivo* study of a certain compound, several assays can serve as indicators of its absorption, distribution, metabolism and elimination (ADME) fate upon administration. From these, it is possible to adequate certain structural aspects or assess other administration routes or formulations to achieve the desired pharmacokinetic properties. In here, compound **815A**'s octanol/water partition coefficient (*logP*), plasma stability, membrane permeability (parallel artificial membrane permeability assay – PAMPA) and plasma protein binding (PPB) were experimentally assessed to confirm some of the data previously estimated and also to correlate with the observations made so far. The obtained results are summarized in Table 3.

Table 3: *In vitro* ADME assays results.

Assay	Result
logP value	-0.25 ± 0.03
Plasma stability	Stable
PAMPA	Not permeable
PPB	High affinity

Overall, the predictions previously made were confirmed. Compound **815A** has a negative *logP* value, indicating that it has a higher affinity for aqueous medium, as demonstrated by the several tests here described. This is also in accordance with the PAMPA results, confirming the predictions made regarding the compound low probability of easily permeate lipidic membranes, corroborating the predictions suggesting that the test compound would be poorly active if orally administered, ratifying the necessity of developing an injectable formulation. Compound **815A** was also found mostly stable to the activity of plasma enzymes and shows a high affinity to plasmatic proteins, being, therefore, expected to remain in circulation, at a concentration equilibrium between its free form and bonded to proteins, until clearance, upon IV administration.

2.5. Controlled-release formulation development

Controlled-release drug delivery systems are designed to release the API at a predictive rate and for an extended period of time. These are usually developed to ensure maximal drug efficacy, minimal side effects and drug level fluctuations, whilst enhancing the patient's compliance to the therapeutics.[22,23] Particulate systems have been widely exploited for parenteral controlled-release systems, specially polymeric systems due to its biocompatibility and biodegradable characteristics.[24] The one polymer that has attracted most attention is poly-lactic-co-glycolic acid (PLGA), which is approved by the competent entities for drug delivery systems in humans.[22,24] PLGA's hydrolysis releases the monomers lactic and glycolic acids, easily metabolized by the human body within the Krebs cycle.[25] Besides its biocompatibility, the extensive research conducted on this polymer, allowed the conclusion that PLGA enables the formulation of this kind of systems for several types of drugs (either hydrophobic and hydrophilic), protecting them from being degrading whilst prolonging its release (Figure 6). Moreover, systems with modified-surface particles enabling a targeted release have also been developed.[25] Another aspect highly beneficial from this co-polymer is its variable nature, meaning that the polymer's physicochemical properties depend on the lactic and glycolic acid content. For instance, a higher content of lactic acid, the slower the rate of disintegration of the particle will be.[26]

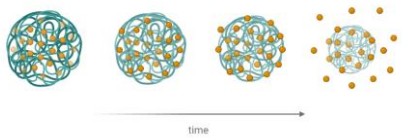

Figure 6: PLGA nanoparticle degradation through time in systemic circulation along with the drug's release.

The standard method to prepare PLGA-nanoparticles (NPs) is the emulsification-solvent evaporation technique, namely the double emulsion (W/O/W) variation, which allows the production of nanospheres for hydrophilic compounds.[24-26] The NPs production optimization was conducted by giving special attention to the final particles' size as well as the polydispersity index (PdI), which is a measure of the sample's size heterogeneity. For a suitable IV administration with a long-circulation behavior, the size range of NPs is believed to be between 10 and 200 nm with a PdI value equal or bellow 0.2.[27,28] During the production process optimization it was found that using higher sonication intensities (50% amplitude) leads to smaller NPs, and that both the addition of a final lyophilization step and preparing the NPs suspension at a 0.25 mg/mL concentration prior to analysis have positive effects in the PdI. According with the literature, bigger NPs are obtained when increased the amount of polymer used. However, in here, the opposite was observed (Figure 7).[29,30]

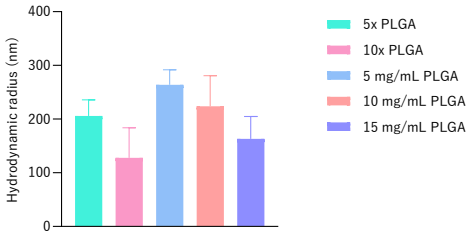

Figure 7: Effect of the PLGA concentration in the final NP size. Results presented of the mean ± SD hydrodynamic radii values with the correspondent standard deviation, of triplicates. NPs samples prepared at a 0.25 mg/mL prior to measurement.

More studies regarding the effect of the PLGA concentration in the NPs size within the defined protocol will have to be conducted. Nevertheless, despite not being able to obtain NPs with the desired PdI values, particles produced using the 10x PLGA condition with higher sonication intensities and a freeze-drying step were within the desired size range (128 nm). Therefore, NPs produced this way were studied for their ability to release compound **815A** in a controlled fashion. Initially, a long-term release assay was setup. This involved the production of large amounts of NPs using the defined method, and their incubation in an adequate medium to assess the release of compound **815A** over time, being the release of test compound monitored by HPLC analysis of the liquid phase. The dissolution of NPs over time was easily observed when these were pelleted in the tubes each day, without only a few solid residues being observed after 5 days of incubation, in contrast to the large solid pellet observed in the beginning. Despite that, no compound **815A** could be detected in the samples' supernatants. This was thought to be a direct consequence of the low sensitivity of the developed HPLC method. Nevertheless, because it became obvious that the NPs were being dissolved, and considering that when this happens, their content is released, an efficacy experiment was designed and conducted to evaluate the usefulness of this system in controlling the activity of NK cells.

2.5.1. PLGA-NPs efficacy

As mentioned before, compound **815A** is efficient in inducing the activation of NK cells within specific concentration intervals. One of the indicators of NK cell activation is the secretion of cytokines, in particular IFN-γ.[31,32] One important aspect is the fact that concentrations of **815A** above the defined range prevent NK cell activity and IFN-γ secretion, as mentioned before (see Section 1). Therefore, it is crucial that the amount of **815A** in circulation remains within the defined concentration range to achieve optimal NK cell activity for a prolonged period. As no information regarding the degradation kinetics of PLGA NPs could be obtained, an indirect approach was attempted. In this, stirred cultures of peripheral blood mononuclear cells (PBMCs) were incubated for 5 days with the free **815A**, **815A**-loaded NPs, or unloaded NPs, produced as described before. Supernatant samples were collected from each condition at regular timepoints and assayed to detect and quantify the levels of IFN-γ (Figure 28).

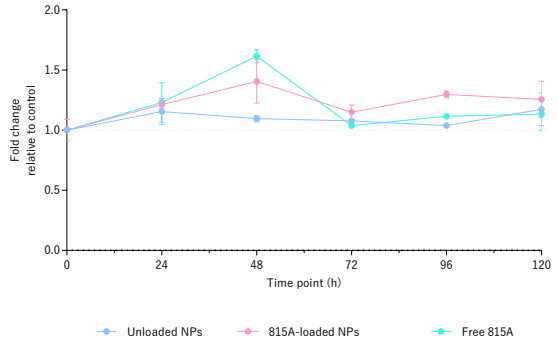

Figure 8: Quantification of IFN-γ in the supernatants of PBMC cultures from donor D, treated with 815A-loaded NPs, unloaded NPs or free 815A, over a 5-day period. The levels of IFN-γ were determined by ELISA in duplicate and normalized to the time point of 0 hours. Values presented are the mean ± SD of two replicates per condition.

In this assay, unloaded NPs were used as control to confirm the biocompatibility of the material and the NPs, as some may be able to induce cytokine release upon contact with immune cells.[33] As observed, the addition of unloaded NPs to the cell culture seems to have increases slightly the production of IFN-γ, but this increase can be due not only to the addition of the NPs but also to the introduction of cells into an artificial culture system. On the other hand, **815A**-loaded NPs present a much more pronounced effect on the secretion of IFN-γ, reaching quite high levels after 48 hours. Strikingly, the amount of IFN-γ in the supernatant remains mostly constant for the remainder of the experiment. In contrast, the culture treated with the free form of **815A** also presents a peak concentration of the IFN-γ after 48 hours of treatment, but the level of this cytokine falls to basal levels immediately after. The fact that **815A**-loaded NPs seem to be able to maintain the activity of NK cells over an extended period of time suggests that the compound is being released in a controlled fashion. Because, contrarily to what is observed with the free form of **815A**, the levels of IFN-γ are kept steady, one can hypothesize that the **815A**-loaded NPs are releasing the compound at a steady state that allows the maintenance of its concentration neat the therapeutic window. However, a larger set of experiments, donors and conditions is necessary to confirm this.

3. Conclusions

The major goal of the work here conducted was to develop an IV formulation of the small organic molecule **815A** that induces the cytolytic activity of NK cells. Several properties of this molecule had to be studied before developing the injectable formulation, especially considering that compound **815A** has a poor solubility profile. Despite containing structural features that should allow the easy dissolution of this molecule in aqueous medium, previous work conducted in the research group, concluded that stock solutions of compound **815A** had to be prepared in an organic solvent (DMSO) before dispersion in water. Although efficient to some point, solutions prepared by this method would often present precipitated and only relatively low concentrations of this could be prepared and stored. Moreover, the presence of DMSO in a formulation was undesirable due to its possible toxic effects. A large set of strategies are available to increase the solubility of APIs for administration via parenteral routes. The simplest encompass adjusting the solution's pH or adding either a co-solvent or a surfactant, whereas the more complex ones evolve the formation of complexes with cyclodextrins or using dispersion systems. Adjusting the solution's pH and using co-solvents were disregarded in this work since the test compound's pKa values were unknown. Thereby, the use of surfactants was exploited being found that sugars and alcohol sugars could easily stabilize and aid the solubilization of the test compound in aqueous media. To try to produce a solid and readily soluble formulation, all **815A**/sugar or sugar alcohol mixtures were dried, removing the co-solvent (DMSO) and resolubilized in water. From all the formulations tested, the mannitol-based ones produced free flowing powders that could be easily dissolved producing clear solutions. Moreover, these were found stable over time, without any visible clouding or precipitation of any of the mixture's components. This is in line with the wide use of mannitol a stabilizer of lyophilizates and biopharmaceutical active substances.[34,35] Fine tuning of the formulation involved varying the proportions of compound **815A** stock solution and the 5% mannitol solution, being concluded that the drying step efficacy and the ease of the following dissolution and handling steps are directly correlated with the amount of aqueous mannitol phase. Therefore, a 1:20 proportion of **815A** stock solution to 5% mannitol was chosen as final free form formulation of the test compound. The solubility issues of compound **815A** did not only influenced the formulation development but also its separation and quantification methods. Several chromatographic conditions were tested, but none was found efficient in separating compound **815A** from complex matrices. For instance, in a C18 column the non-polar moieties of the compound did not enable its adsorption to the column, eluting along with the sample's vehicle, and effect that was also observed when using a polar stationary phase. Despite of being constituted by a large number of hydrophobic moieties, compound **815A** does not seem interact much with non-polar surfaces. Corroborating this, are the *in vitro* ADME assays conducted to determine its *logP* value and plasmatic membrane's permeability. Both assays indicated that the compound has higher affinities towards aqueous media, surprisingly contrasting with the solubility issues previously mentioned. This dual behavior difficulted the task of finding the right chromatographic conditions to better isolate and quantify the test compound. At this point, the possibility of separating the **815A** counting on its permanent cation through ion exchange chromatography was assessed. The method used involved the modification of a C18 column surface with anionic detergents. However, this approach was also fund unable to retain the test compound, besides being too laborious and poorly reliable. It was thus understood that none of the assessed chromatographic conditions enabled both its separation and quantification in HPLC in reproductible, efficient and selective way. Therefore, the development of a methodology capable of selectively isolate the test compound from biological samples prior to its quantification had to be developed. This involved the use of a plasma surrogate spiked with test compound. This was submitted to different deproteinization techniques aiming at simplifying the sample and recovering compound **815A**. However, after the protein removal step, no compound, or very low amounts of it, were observed in the supernatant. It was hypothesized that the compound was being removed along with the proteins due to its probably high affinity for them. This was assessed by comparing the data obtained in plasma surrogate samples with the ones obtained using control samples without any protein content. Whereas in the control samples a recovery of 99% of the initial amount of compound **815A** was observed, no compound was detected in the supernatant of protein-containing samples. The tendency of this compound to bond to plasma proteins was confirmed by digesting the protein pellet and confirming the presence of the test compound in it, and afterwards by conducting an equilibrium dialysis assay. In both cases it was concluded that the compound **815A** has a high PPB tendency, as predicted before based on its structural features. The only deproteinization technique that allowed the isolation the test compound from both plasma surrogates and real plasma samples was the addition of high concentrated salt solutions. Surprisingly, instead of causing protein precipitation, the salt addition resulted in the selective precipitation of the compound **815A**. From all the salts tested, better results were achieved with sodium fluoride. This allowed an 80% recovery of the compound from blood plasma samples. Efficacy that is maintained upon use of the soluble formulation with mannitol. After optimization, this isolation process comprised the addition of a saturated solution of sodium fluoride after the sample's incubation with a potent protease, found to aid on the compound's extraction. Once collected, the final pellet had to be dissolved for further quantification in a C18 column eluted at 1 mL/min with a 1:1:1 mixture of 0.1% aqueous formic acid – acetonitrile with 0.1% formic acid – methanol. There was still a lack of interactions between the compound **815A** and the stationary phase, however these were the only conditions allowing the observation of a well resolved and isolated peak. The method was further refined with the use of an internal standard to reduce the inter-assay variations caused by the injection system. At this point, all the aims initially traced for this project were completed. A water soluble formulation of compound **815A** had been developed, as well as a protocol that allowed both its isolation and quantification in biological samples. However, considering the known dual immunomodulatory behavior of the NKp30 receptor, another formulation approach was assessed with the aim of achieving a prolonged and effective activation of NK cells. For that, a controlled-release drug delivery system with biocompatible and biodegradable features was developed. The double emulsification-solvent evaporation technique (W/O/W) was chosen to produce **815A**-loaded polymer (PLGA) nanoparticles. As these NPs were intended to be used in IV

formulations, their size would have to be within the range of 10 to 200 nm, with a PdI value below 0.5. Although NPs with hydrodynamic radii averaging 118 nm could be produced by the developed methods, none of the attempts results in samples with PdI below 0.5. Despite this, the effect of encapsulating the test compound in NPs on the activation of NK cells was evaluated using primary cultures of PBMCs. In these, the biocompatibility of PLGA nanoparticles was confirmed, as no increases in the secretion of the cytokine IFN-γ were observed in the culture treated with unloaded-NPs. On the other hand, in the culture treated with **815A**-loaded NPs, an increase in the levels of secreted IFN-γ could be observed after 48 hours. More important, this effect persisted until de end of the experiment (day 5), with the level of IFN-γ in the medium remaining stable. In the control culture, treated with an equivalent amount of **815A** in its free form, the effect dropped immediately after reaching the peak at 48 hours. This suggests that the use of nanoparticles to sustain a controlled and prolonged release of compound **815A** could, in fact, result in a more stable and efficient activation of NK cells, increasing their efficacy in tumor targeting and lysis.

3.1. Critical overview and perspectives

Overall, the results here presented answer some of the questions regarding the behavior of compound **815A** in biological medium. The ADME properties here determined are in accordance with the predicted, especially in terms of *logP*, membrane permeability, plasma stability and plasma protein binding. Regarding the selective extraction and quantification procedures developed herein, some aspects still require further development. The HPLC quantification method presents a low sensibility encompassing high LOD and LOQ values which can highly influence and jeopardize the performance of several assays requiring the use of low concentrations of the test compound. This problem is further enhanced by the necessity of performing the prior isolation of compound **815A** from biological samples, increasing the associated errors. In this work, the use of a C18 column was preferred over the CN column due to the lack of repeatability of analysis performed in the later. However, these were probably caused by matrix effects that could be minimized with the extraction procedure here developed and the use of an internal standard. However, this would still require a great deal of optimization of both the extraction of compound **815A** and of the chromatographic conditions. nevertheless, the low LOQ and LOD values observed with the CN column are quite attractive, and its use should be revisited in the future. The results obtained in the NK cell stimulation assay using **815A**-load NPs provide valuable insights into the use of a modified release system for the delivery of controlled amounts of the test compound into the blood stream. The fact that, in the assays here performed, NK cell activity seems to be sustained over a longer period of time, in contrast with what is observed using the free form of **815A**, grants this method great attractiveness. The work here developed should encourage the further development of a NK cell-based therapy using compound **815A**, promoting the strong and controlled activation of NK cells against cancer.

4. Materials & Methods

4.1. General procedures and equipment

4.1.1. Reagents and solvents

All reagents were used without any purification procedures. Compound 815A was obtained from the synthesis line of the –Design, synthesis, and toxicology of bioactive molecules (BIOMOL) group of Centro de Química Estrutural (CQE), as a dry powder with a purity greater than 98%, as determined by nuclear magnetic resonance (NMR) titration experiments and liquid chromatography – mass spectrometry (LC-MS). All solvents used in the HPLC system were of gradient grade (Thermo Fisher Scientific, Waltham, MA, USA). All other reagents and solvents were of p.a. grade or higher.

4.1.2. High Performance Liquid Chromatography

A. Chromatographic system

HPLC was conducted on an Ultimate 3000 Dionex system consisting of an LPG-3400A quaternary gradient pump and a diode array spectrophotometric detector (Dionex Co., Sunnyvale, CA, USA) and equipped with a Rheodyne model 8125 injector (Rheodyne, Rohnert Park, CA, USA). HPLC analyses were performed with a Luna C18 (2) column (250 mm × 4.6 mm; 5 µm; Phenomenex, Torrance, CA, USA), at a flow rate of 1 mL.min^{-1} using different mobile phases and elution sequences. The optimized conditions consisted of a 10-minute isocratic elution of aqueous 0.1% formic acid – acetonitrile with 0.1% formic acid – methanol, at a proportion of 1:1:1. All samples were prepared in DMSO with or without 10 mM anthranilic acid as internal standard. Each injection admitted 10 µL of sample into the system through the µL-pickup mode using isopropanol/water (9:1) as pickup fluid. Chromatograms were recorded between 220 and 700 nm, using the fixed wavelengths of 254 or 330 nm for monitoring.

B. HPLC columns

The HPLC columns used in this work were:

- Luna C18 (2) column (250 mm × 4.6 mm; 5 µm; Phenomenex)
- Luna C8 (2) column (250 mm × 4.6 mm; 5 µm; Phenomenex)
- Microsorb-MV 100-5 CN (250 mm × 4.6 mm; 5 µm; Varian, Palo Alto, CA, USA)
- XBridge BEH Amide XP 130Å (2.1 mm X 150 mm; 2.5 µm; Waters Corporation, Milford, MA, USA)
- All columns were protected with a pre-column (SecurityGuard Cartridge C18 4x3.0 mm; Phenomenex).

C. Column functionalization for cation exchange chromatography

For cation exchange chromatography experiments, a C18 column (Luna C18 (2) column (250 mm × 4.6 mm; 5 µm) was functionalized with either 10 mM of sodium dodecyl sulfate or 2 mM octanoic acid in water, flowing at 0.3 mL.min^{-1} for 3 hours.[83] Excess surfactant was removed by running the system with water at 0.6 mL.min^{-1} for 2 hours. The column was recovered by removing all the surfactant running methanol for 24 hours at 0.1 mL.min^{-1}.

4.1.3. Ultraviolet-visible Spectroscopy

The UV-Vis spectra acquired throughout this work, either for quantitative and/or qualitative purposes, were recorded using a SPECTROstar Nano microplate reader equipped with an *LVis* low-volume measurements plate (BMG Labtech, Ortenberg, Germany).

4.1.4. Zetasizer

The NPs hydrodynamic diameters and polydispersity indexes (PdI) were measured using a Zetasizer (ZEN3600 Nano ZS; Malvern Instruments, Worcestershire, UK). Measures were performed in 1 cm polycarbonate cuvettes, at 25 ºC.

4.1.5. Melting point measurements

The melting point determination was conducted in a Buchï B-545 (Buchï AG, Flawil, Switzerland) using a Ø1.0 mm glass capillaries, without correction. The maximum measurable value was 400 ºC, with an error of 0.8 ºC.

4.2. Study of ADME properties

4.2.1. Isolation of compound **815A** from different matrices

The fully optimized isolation protocol encompassed the pre-incubation of the sample with 0.4 mg/mL of proteinase K (Cat# A4392; PanReac Applichem, Darmstadt, Germany) and 5 mM of calcium chloride for one hour at 37 ºC (this step is suppressed or samples without protein), followed by the addition of five times the volume of the initial sample of a saturated solution of sodium fluoride (Cat# A13019; Alfa Aesar, Haverhill, MA, USA), followed by incubation at 3 ºC overnight. Samples were then 16000 x *g* at 4 ºC for 5 minutes. The supernatant was discarded, and the pellet washed with the same volume of saturated sodium fluoride solution and water. The pellet obtained in the end was either dried or dissolved for subsequent analysis.

4.2.2. Plasma stability assays

Freshly isolated human blood plasma samples (obtained as detailed in Section 4.4.1) were spiked with 2 mM of compound **815A** in DMSO. All samples were incubated at 37 ºC for three consecutive days. Aliquots were collected at different time points throughout the incubation period and submitted to the developed and optimized isolation and quantification processes (Sections 4.2.1 and 4.1.2).

4.2.3. *logP* determination

Six tubes containing 1 mL of an aqueous solution of 1 mM of **815A** in 5% mannitol (pH 7) were prepared. To three of these, 1 mL of octanol was added. All tubes were vigorously vortexed for 1 minute and then left agitating at room temperature for 2.5 hours. The aqueous portion of each sample was collected and the concentration of compound **815A** was determined by spectrophotometry (Section 4.1.3).

4.2.4. PAMPA assays

The PAMPA assay (Parallel artificial membrane permeability assay) was conducted using artificial lipidic membranes assembled in filter plates (MultiScreen IP Filter Plate, 0.45 µm; Merck KGaA, Darmstadt, Germany) using a mixture of dipalmitoylphosphatidylcholine:stearic acid (80:20, w/w; Avanti Polar Lipids, Inc., Alabaster, AL, USA). The artificial lipidic membrane was prepared by adding 4 µL of the lipid mixture to each well, being the correct application evidenced by the change in transparency of the membrane. Acceptor wells were filled with 320 µL of 50 mM ammonium bicarbonate buffer pH 7.4, and the donor wells were filled with 150 µL of 1 mM **815A** solutions in 5% mannitol. The plaque was incubated for 18 hours at room temperature. Thed content of both acceptor and donor wells were subjected to analysis to identify and quantify compound **815A**.

4.2.5. Plasma protein binding assays

D. Protein precipitation

Plasma surrogate (40% BSA solution) and water samples were spiked with 0.2 mM of compound **815A**. After being gently mixed, five volumes of absolute ethanol were added to both. All samples were centrifuged at 16000 x *g* for 5 minutes at room temperature and the supernatants were collected and stored. To the resultant pellets, 0.4 mg/mL of proteinase K and 5 mM of calcium chloride were added, and the samples were left incubating for one hour at 37 ºC. All samples, including the supernatants collected before, were vacuum dried before redissolution in DMSO for quantification (Section 4.1.2).

E. Dialysis

The equilibrium dialysis procedure was conducted using a dialysis tubing with a molecular weight cut-off of 10 kDa (D9652; Sigma-Aldrich, St. Louis, MO, USA). The interior of the membrane was filled with 5 mL of blood plasma spiked with 0.1 mM of compound **815A** and submerged in 5 mL of a 5% mannitol solution. The system was left softly stirring for 3 days at room temperature. The mannitol solution was collected and subjected to the isolation protocol (Section 4.2.1) and then quantified by HPLC (Section 4.1.2).

4.3. Intravenous formulations

4.3.1. Free form formulation

The injectable formulation of compound **815A** in its free form was prepared using mannitol as stabilizer. Several stabilizers and proportions were tested following the generic procedure described below. A sample of compound **815A** dissolved in DMSO was added to twenty times its volume of a 5% mannitol solution. The resultant clear solution was filtered through a 0.22 µm syringe filter into sterile vials and concentrated for 48 hours in a centrifuge concentrator, yielding a white to off-white powder readily soluble in water.

4.3.2. Controlled release system formulation

F. Preparation of nanoparticles

PLGA nanoparticles were prepared routinely using the general procedure described below.

A 10 mg/mL PLGA (poly(lactic-co-glycolide4) 50:50, 5000-10000 Da; Acros Organics, B.V.B.A, Geel, Belgium) solution in ethyl acetate was mixed with a third of its volume of the free form formulation of compound **815A**. The mixture was emulsified using an ultrasonic homogenizer coupled with a MS 72 probe (Bandelin Sonoplus, Berlin, Germany) with a 50% amplitude defined, for 3 minutes. Thereafter, the primary emulsion (W/O) was added to another tube containing an equal volume of 5% mannitol and, once more, was well mixed and sonicated. This secondary emulsion (W/O/W) was added drop wise into three times its volume of stirring 5% mannitol and, subsequently, left overnight at 400 rpm at constant temperature (30 ºC) to evaporate all the solvent (HLC Heating-ThermoMixer MHR 2; Ditabis AG, Pforzheim, Germany). The suspended NPs were collected after consecutive centrifugation/washing steps with deionized water at 4000 x *g* for 20 minutes. Lastly, the NPs were resuspended in 2-3 mL of 1% Sucrose and freeze-dried (Alpha 1-2 LDplus; Christ, Osterode am Harz, Germany).

4.4. Biological assays

4.4.1. NK cell activation assays

The presented protocol follows all recommendations of the Institutional Ethics Committee (process 15/2021 CE-IST). All participants provided a written informed consent and personal data protection was safeguarded in all instances.

G. Blood collection

Peripheral blood was obtained by venipuncture of the median cubital vein of four volunteers (Table 4) using a closed S-Monovette® system (Sarstedt, AG & Co. KG, Nümbrecht, Germany). Blood was collected into tubes containing EDTA or clot activator, for cell and serum isolation, respectively.

Table 4: Blood donors' characteristics.

Donor	Gender	Age (years)	Weight (kg)
A	Male	25	68
B		40	72
C		33	85
D	Female	23	74

H. Blood serum isolation

To isolate blood serum, blood collected into clot activator tubes was left at room temperature for 30/45 minutes before centrifugation at 1500 x *g* for 10 minutes. The serum layer was carefully separated from the clot and stored at -20 ºC.

I. Blood plasma and PBMCs isolation

Blood collected into EDTA tubes was layered onto an equal volume of Ficoll Histopaque®-1077 (Cat# 10771; Sigma-Aldrich) in 50 mL conical sterile tubes. After a 30-minute centrifugation at 400 x *g*, the upper layer of plasma was transferred into a sterile tube, filtered through a 0.22 µm syringe filter and immediately frozen. The interphase containing the peripheral blood mononuclear cells (PBMCs) were transferred into a 50-mL tube and immediately diluted with sterile PBS. Cell suspensions were centrifuged at 300 x *g* for 10 minutes and the pellets washed with 40-45 mL of sterile PBS at least two times to remove Ficoll and platelets.

J. PBMC cultures

PBMCs isolated as described before were resuspended to a density of $1x10^6$ cells/mL in culture medium comprised of a 1:1 mixture of DMEM (Cat# D5523; Sigma-Aldrich) and Ham's nutrient mixture F12 (Cat# N3520; Sigma-Aldrich) base media, supplemented with 5% autologous serum (isolated as described in Section H), 15 µM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cat# A1069; PanReac Applichem), 100 U/mL penicillin and 100 µg/mL streptomycin (Cat# P4333; Sigma-Aldrich), 24 µM β-mercaptoethanol (Cat# M3148; Sigma-Aldrich), 20 mg/L *L*-ascorbic acid (Cat# A4544; Sigma-Aldrich), 5 µg/L sodium selenite (Cat# S5261, Sigma-Aldrich), 0.25 µg/mL Amphotericin B (Cat# 17-836E; Lonza, Basel, Switzerland), and 250 U/mL of rhIL-2 (Cat#200-02; Peprotech, London, UK).

The cell suspension was equally divided in three sterile Erlenmeyer flasks (approx. 30 mL per flask) equipped with cotton plugs. To each, either unloaded NPs, **815A**-loaded NPs or **815A** in DMSO stock solution, were added. The amounts of **815A**-loaded NPs and **815A** stock solution were adjusted to yield a final maximum concentration of test compound of 5 µM. The controlled flask (unloaded-NPs) received the same amount of NPs as the **815A**-loaded NPs' flask. All systems were incubated at 37 ºC in an orbital stirring platform (100 rpm). Samples of each flask (1 mL each) were collected every 24 hours and stored at -20 ºC after a first centrifugation at 200 x *g* and the second at 10000 x *g* both for 10 minutes at room temperature.

K. ELISA assays

IFN-γ quantifications were performed using a commercial ELISA kit (Human IFN-gamma DuoSet ELISA, Cat# DY285B; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Colour development was achieved using the Substrate Reagent Pack (Cat# DY999; R&D Systems) and measured at 450 nm with correction at 570 nm in a plate reader (Section 4.1.3).

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