

Present and Future Application of Nanoparticle Based Therapies in B-Chronic Lymphocytic Leukemia (B-CLL)

Eduardo Mansilla^{1,2}, Gustavo H. Marin², Luis Núñez³,
Gustavo Larsen⁴, Nelly Mezzaroba⁵ and Paolo Macor⁵

¹National University of La Rioja, UNLAR, La Rioja,

²CUCAIBA, Ministry of Health, La Plata, Buenos Aires

³University of Chicago, Chicago, Illinois, USA and Bio-Target, Chicago, Illinois,

⁴LNK Chemsolutions, Lincoln Nebraska, USA and Bio-Target, Chicago,

⁵Department of Life Science, University of Trieste,

^{1,2}Argentina

^{3,4}USA

⁵Italy

1. Introduction

We describe a variety of polymer biodegradable nanoparticles (BNPs) that can be created in an attempt to find an effective and durable treatment for B-Chronic Lymphocytic Leukemia (B-CLL). Many different drugs including those like Chlorambucil (CLB) that has been the gold standard B-CLL's chemotherapeutic treatment preference for years (1), or even those not traditionally considered or used as antineoplastic agents like Hydroxychloroquine (HCQ) (2), can safely be encapsulated inside nanoparticles and specifically be targeted to the selected tumor cells by the coating of monoclonal antibodies in their surface (3,4). In this way all these drugs could be released in a steady manner exclusively into the desired neoplastic cells. This would give several advantages in relation to traditional drug delivery methods as a significant less toxicity is produced in non cancer cells while very high concentrations of the therapeutic compounds with great apoptotic effect are reached only at the targeted selected B-CLL cells level.

Therapeutic systems of this kind are relatively easy to produce in the large scale, are probably very safe, and will elicit a negligible immune response (3). BNPs like the ones we have developed, will offer a great promise as non-viral biocompatible and biodegradable vectors and carriers of drugs, peptides or other substances, with targeting capacities to specific cell sites by surface receptors of monoclonal antibodies (mAbs). Our data indicate that these nanoparticles with surface mAbs are suitable as a selective drug delivery method to treat B-CLL, other lymphomas and probably autoimmune disease such as Rheumatoid Arthritis and Lupus Erythematosus between many others. When loaded with the lysosomotropic agent HCQ alone, or combined with CLB, they elicited a strong apoptotic effect (3) (4). Additional data revealed that these BNPs were non-toxic for healthy animals,

and had prolonged an outstanding survival in mice models of human lymphoma and B-CLL. There is a real need to comprehend and define all the basic processes needed in order to commercially produce viable products of this kind. This includes the present knowledge that has been acquired in the development and use of nanoparticles for the cure of B-CLL as well as all the possibilities that have been opened by their introduction, such as regulatory/safety, environmental, health and societal implications of these new treatments (5).

2. B-CLL and the need for new therapeutic approaches: An opportunity for nanoparticle systems

B-CLL is the most common form of leukemia in the western world. It results from a relentless accumulation of small mature monoclonal lymphocytes. Following a recent demonstration of a significant increase in the proliferative pool of CLL cells *in vivo*, the gradual accumulation of malignant B-CLL cells seems to be primarily the consequence of their selective survival advantages relative to their normal B-cell counterparts (6). As the disease is mainly caused by defective apoptosis it is thus a good candidate for treatment by pro-apoptotic agents. Even though a large amount of research has been done during the last past years, the prognosis has not changed (7). A major problem with treating patients with cancer and B-CLL by traditional chemotherapeutic regimes is that their tumors often develop a multidrug resistant (MDR) phenotype and subsequently become insensitive to a range of different chemotoxic drugs. One cause of MDR is overexpression of the drug-effluxing protein, P-glycoprotein. It is now apparent that P-glycoprotein may also possess a more generic antiapoptotic function that protects P-glycoprotein-expressing cancer cells and normal cells from death (8). B-CLL cells with unfavorable cytogenetic alterations such as deletion of chromosome 17p with loss of p53 are often resistant to fludarabine and cyclophosphamide (9,10). Similarly, CLL cells from patients with advanced disease stages or having a history of prior chemotherapy, exhibit elevated oxidative stress (11) and thus may have a greater potential to acquire additional mutations and genetic abnormalities, leading to drug resistance and disease progression.

3. The history of the first biodegradable nanoparticle system for the treatment of B-CLL and lymphomas that could also work for autoimmune disease

By the end of 2007 Dr. Luis Núñez, a biochemist that founded with Dr. Gustavo Larsen, Bio-Target, a Chicago, USA, start-up nanotechnology company, with a new interesting intellectual property in the production of biodegradable nanoparticles that could be loaded with many drugs and coated with monoclonal antibodies, contacted Dr. Eduardo Mansilla in La Plata, Argentina, and agreed to develop together ideas and products in this direction. By that time Dr. Mansilla was very involved in B-CLL research, and for many years looked for a system that could deliver HCQ in enough concentrations inside tumor B-CLL cells. In this situation the technology offered by Bio-Target seemed to him the wright one to use. In less than six months the research group in Argentina of which Dr. Gustavo H. Marin was also intensively participating, had the particles ready and the *in vitro* testing done with superior results, having the anti-CD20 antibody Rituximab coated in the surface of the BNPs. The nanoparticles were specifically attaching to the B-CLL cells and as they were penetrating them, an outstanding

apoptotic process was seen with more than 95% killing effect in less than 48 hrs. In this way, we produced and tested in vitro in an amazing fast time the first biodegradable nanoparticle system in the history of medicine with a non-traditional antineoplastic old drug such as HCQ and a monoclonal antibody approved by the FDA, Rituximab, for the treatment of B-cell malignancies, with very good efficacy. We did some further testing with particles coated with the anti-CD19 mAb and its combination with the anti-CD20, as well as mixtures of HCQ and CLB. Then, we offered our technology to the Italian group, from Trieste, conducted by Dr. Paolo Macor in order to do more in vitro testing and a large animal study in a mouse model of Burkitt's lymphoma. The results were reproduced in the same manner with similar results in vitro. The animal study was a great surprise, as almost all animals treated with the nanoparticle system were alive after more than 120 days, while the control group was all dead by that time. After that, we started conversations with United States and European research groups to introduce this technology into further more animal studies not only for B-CLL and Lymphoma but also for autoimmune disease, specially SLE and Reumathoid Arthritis, as well as a human clinical trial for B-CLL patients. At this time, it is clear that the developmental steps of this new technology was successful and done in just a few months, later on, the industrialization and approval by regulatory agencies, as well as the commercialization and final benefit of the patients is taking an unacceptable but predictable long time and delay. These new therapeutic strategies are really urgently needed, especially because they could easily switch on new apoptotic responses and restore sensitivity to drugs in B-CLL cells. In this way, nanoparticle-based "smart" therapeutics will generate both evolutionary as well as revolutionary products in the near future for B-CLL. There is enough evidence now, in order to think that these systems will profoundly impact the next generation of treatments for this disease and probably others. If this is to happen, there will be a few key biological requirements for such technologies to be introduced and routinely used by the onco- hematology community. All these aspects, specially related to their design, delivery capacity and their tremendous selectivity in their targeting to specific B-CLL cell sites, is urgently needed to be addressed.

4. Old drugs re-discovered to be used inside BNPs for B-CLL

CLB, which belongs to a family of drugs known as alkylating agents, has been in use for decades to treat hematological malignancies including B-CLL (12). This drug is given orally, which is normally an advantage but in this case, causes problems because the rate at which the drug is absorbed into the bloodstream can vary tremendously from patient to patient (13). Drug developers have tried a variety of techniques to offer new forms of delivery of this interesting old drug, but each of these methods has proven less than optimal (14). Now, however, we have created these BNPs that appear to could solve these issues and hold the promise of improving the utility of CLB not only in B-CLL but in many other cancer therapies. This could also be true for many other old onco-hematologic chemo-therapeutic agents with a fairly interesting efficacy and safety profile that could be re-discovered for their use in B-CLL and other leukemias and lymphomas by delivering them in nanoparticles. This could be the case of doxorubicin or tamoxifene (15,16), or even bendamustine. This last drug has been used for more than 30 years in the treatment of lymphoma, but little is known about the optimal dosing schedule in relapsed or refractory B-cell chronic lymphocytic leukemia (CLL). Various dose and treatment schedules have been used empirically, and several studies have shown impressive efficacy specially in heavily pre-treated and treatment-refractory patients (17,18).

5. Non-classical drugs for the treatment of B-CLL: HCQ, magnolol, honokiol, parthenolide, phenylethyl-isothio-cyanate (PEITC) and others delivered in BNPs

Many compounds non-traditionally used as anti-neoplastic agents have been shown to put cancer cells into pro-apoptotic programs and could be very useful for B-CLL treatment. A great diversity of still undefined compounds with anti-cancer properties can be obtained from botanical species. The pharmaceutical industry has been substantially but slowly scaling-up research efforts and partnering up with research universities for finding natural herbal and natural alternatives to fight cancer instead of the conventional expensive and tedious large scale process of screening thousands of synthetic compounds to find a final cure or solution to cancer. Scientists have given proof of the valuable anti-inflammatory, antioxidant, and cholesterol-lowering benefits of resveratrol, curcumin, and green polyphenols, natural compounds between many other that are found in red wine, curry, and green tea extracts respectively (17,18). Some of these natural products could be effective in B-CLL by activating different cell death pathways. The active principles of a group of very well known herbs like *tanacetum parthenium*, *magnolia grandiflora*, cruciferous vegetables, turmeric, and many others, have been previously described as components of different Japanese, Latin American or Chinese traditional medicine having recognized anti-angiogenic and/or anti-tumor properties (19). Many of the difficulties found in human application of these drugs have been related mainly to bioavailability and toxicological issues. In this way and considering that these drugs are usually very cheap and can be obtained from botanical species in unlimited amounts it is very interesting to speculate in its use in B-CLL by its administration inside nanoparticles. It is an interesting issue that many of these herbs contain parthenolide (PTL) as one of their major active components (20). This last substance is a sesquiterpene lactone, a novel natural NF-kappa B inhibitor with antineoplastic properties (21). In general, parthenolide is well tolerated by humans, making it a good candidate for further clinical testing as an anti B-CLL agent. Obtained mainly from *Tanacetum parthenium*, *Magnolia grandiflora* and other plants, it has recently demonstrated an interesting anti-tumoral activity against CLL, some solid tumors and acute myeloid leukemia (21,22), but it was only tried in B-CLL in a study done by our group (23). We showed for the first time that PTL has a potent apoptotic effect on B-CLL cells without a great impact on normal PBMC (24). PTL displayed potent cytotoxic and apoptotic effects on B-CLL cells in vitro. B-CLL cells treated with PTL resulted in a dose and time dependent cytotoxicity. PTL mediated cytotoxicity occurred at a concentration of 1 μ M and above. A significant decrease in the cell viability of B-CLL cells obtained from 5 patients was seen after one day of culture ($38.1 \pm 6.37\%$) and at 72 h ($90 \pm 5.19\%$) with a PTL concentration of 8 μ M. (Fig. 1). All these responses were dose and time dependent for PTL values from 1 to 10 μ M. By contrast, this compound had little apoptotic or cytotoxic effect in PBMCs of healthy donors even at higher concentrations. These results provided clues for interesting pathways involving different aspects of B-CLL cell apoptosis that could be exploited in therapies with this product. It could be speculated that parthenolide increased the amount of the NF-kappa B inhibitory protein, I kappa B-alpha, and decreased NF-kappa B DNA binding activity. All this evidence suggests that parthenolide may provide an anti-B-CLL effect and could be a potentially effective repertoire for chronic lymphocytic leukemia treatment specially if given in combination with other drugs in nanoparticle systems. Even though all our patients were Rai II and CD38-, compromising mainly a potentially less aggressive category of disease, the

results obtained in this work were more than satisfactory and probably could also be transferred to patients with a poor prognosis. As this compound had little cytotoxic in vitro impact on normal human PBMCs, side effects in the clinical setting could probably be minimized, especially if given inside a nanoparticle system, and this of course, will be a very important aspect to be considered in a chronic disease like B-CLL. It is also possible that a formulation combining parthenolide with some other natural molecules like honokiol or magnolol, obtained indeed from the Magnoliaceae family of plants, or the classical treatments, might have a synergistically beneficial effect in B-CLL, being a potential promising strategy for the treatment of this hematological malignancy.

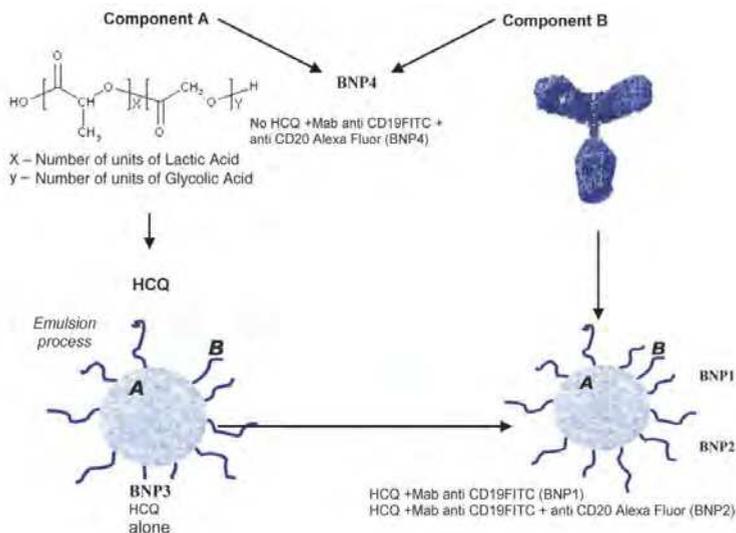


Fig. 1. Targeted biodegradable nanoparticle desing scheme.

Magnolia Grandiflora mainly contains Honokiol, Magnolol and Parthenolide (23) Honokiol and Magnolol are the major active constituents extracted from the bark of different Magnoliaceae species like Magnolia officinalis and Magnolia Grandiflora. They have a variety of pharmacological effects, such as anti-inflammatory, (21) antithrombotic, (25) anti-arrhythmic, (26)antioxidant (27)and anxiolytic effects, (28)and more recently, cytotoxic activity by inducing cell apoptosis in some cell lines (29). Magnolol and Honokiol-triggered apoptotic process is accompanied with down-modulation of Bcl-XL molecules (30) or through Caspase cascades activation (31) We have also shown that an aqueous Magnolia extract displayed a strong apoptotic effect on untreated as well as in heavily CLB treated B-CLL cells in vitro. We have published that Magnolia's extracts have efficacy in apoptosis and cytotoxicity induction and that these properties are exhibited mainly in tumors and not in normal cells, suggesting that an increase in NF-kappa B inhibitory protein and a decrease in NF-kappa B DNA binding activity or EGFR/PI3K/Akt signaling pathway or inhibition of telomerase activity might be involved in apoptosis induction (32). Phenylethyl-isothiocyanate (PEITC) is abundant in cruciferous vegetables, has potent preventing antineoplastic properties as well as pro-apoptotic activities against a large variety of cancers, it is also a ROS-generating agent capable to kill B-CLL cells in vitro and probably in vivo.

6. Hydroxychloroquine for CLL

Concerning HCQ, it could be said that it is an anti-malarial and a Disease-Modifying Antirheumatic Drug (DMARD), very active against rheumatoid arthritis and lupus erythematosus which operates by inhibiting lymphocyte proliferation, antigen presentation in dendritic cells, release of enzymes from lysosomes, release of reactive oxygen species from macrophages, and production of IL-1 (33). Also it has demonstrated to be active as an antiviral agent, since it impedes the completion of the viral life cycle by inhibiting some processes occurring within intracellular organelles and requiring a low pH. For HIV-1, chloroquine and hydroxychloroquine also inhibit the glycosylation of the viral envelope glycoprotein gp120, which occurs within the Golgi apparatus (34). For all of this HCQ, that has been used in the clinic for decades with good results and a very safe profile (35) is a very interesting option to be loaded in nanoparticles with the intention to treat B-CLL and autoimmune diseases and maybe also some viral infections like HIV. Recently HCQ by itself has demonstrated an interesting pro-apoptotic effect and has been selected by the NIH as an anti-cancer drug that deserves further testing. Its antineoplastic properties *in vitro* depend on its concentration but this cannot routinely be obtained *in vivo* by the usual oral route of administration (3). HCQ, induces a decrease in B-CLL cell viability in a dose- and time-dependent manner when tested *in vitro*. The mean LC50 calculated for the cells of 20 patients was 32 +/- 7 microg/ml (range, 10-75 microg/ml). A large increase in apoptotic cell numbers after 24 h of incubation with 50 microg/ml HCQ (55 +/- 6 vs. 23 +/- 3% in medium alone, $p < 0.001$). Indeed, HCQ in leukemic cells induced the features of apoptosis (cell shrinkage, decrease in mitochondrial transmembrane potential, phosphatidylserine externalization, chromatin condensation and DNA fragmentation). HCQ had marked selective cytotoxicity when compared with normal blood mononuclear cells, in which the LC50 was >100 microg/ml at 24 h. HCQ induced the proteolytic cleavage of poly(ADP(adenosine 5'-diphosphate)ribose) polymerase (PARP) and increased the activity of caspase-3. The expression of bcl-2 and bax proteins was significantly modified after incubation with the drug and HCQ activity against CLL cells occurred independently of the presence of IL-4, sCD40L and bone marrow stromal cells (36). The mechanisms behind the effects of HCQ on cancer are currently being investigated. The best-known effects (investigated in clinical and pre-clinical studies) include radiosensitizing effects through lysosome permeabilization, and chemosensitizing effects through inhibition of drug efflux pumps (ATP-binding cassette transporters) or other mechanisms like those of a lysosomotropic agent, meaning that it accumulates preferentially in the lysosomes of cells and in this way promotes apoptosis (37)

7. New treatments

7.1 Monoclonal antibodies

At present, there are mainly two antibodies with great clinical value for patients with CLL. The first is rituximab (Rituximab, Mabthera) that targets the CD20 antigen (38). This CD20 molecule is expressed on almost all B-cells of patients with B-CLL, but the intensity of expression appears to be lower than in patients with non-Hodgkin lymphoma (NHL) (39,40). The second approved mAb is alemtuzumab (Campath-1H), a humanized therapeutic mAb that recognizes the CD52 antigen expressed on normal and neoplastic lymphoid cells. Alemtuzumab is an effective drug in CLL patients with poor risk

cytogenetics, such as deletions in 17p. However, alemtuzumab is ineffective in patients with bulky nodal disease (>5 cm) (41-43). Both these “gaps” of these two mAbs, low CD20 expression and bulky nodal disease, could be overcome by nanoparticles technology. Ofatumumab (HuMax-CD20; Arzerra), is a second-generation, fully human, anti-CD20, IgG1 mAb. Ofatumumab recognizes a different CD20 epitope to rituximab (44,45). Compared with rituximab, ofatumumab has similar antibody-dependent cellular cytotoxicity (ADCC) but stronger complement-dependent cytotoxicity (CDC) and does not induce cell death by apoptosis. Ofatumumab potentially represents an active treatment option with clinical benefit for patients with very poor prognosis who have exhausted standard treatment options but also is a very interesting option for the construction of our particles. Lumiliximab is a genetically primatized, macaque human chimeric anti-CD23 IgG1; mAb investigated for the treatment of relapsed CLL. It induces ADCC and CDC, and enhances apoptosis when combined with current or emerging CLL therapies including CLB, fludarabine, alemtuzumab and rituximab. TRU-016 is an intravenously administered anti-CD37 IgG fusion protein for the potential treatment of B-cell malignancies, including CLL and non-Hodgkin's Lymphoma. In addition, several other mAbs directed against lymphoid cells have been recently developed and investigated in preclinical studies and clinical trials. These treatments include epratuzumab, galiximab and anti-CD40 mAbs (46).

7.2 Phenethyl isothiocyanate-PEITC

Epidemiological studies support the evidence that the consumption of cruciferous vegetables has long been associated with a reduced risk in the occurrence of cancer at various sites, including the prostate, lung, breast, and colon (47).¹ The anticarcinogenic effect of cruciferous vegetables is attributed to organic isothiocyanates (ITCs), which are present in a variety of edible cruciferous vegetables such as broccoli, watercress, cabbage, and so on (47). Phenethyl isothiocyanate (PEITC) is one of the ITC family of compounds that has attracted a great deal of attention owing to its remarkable cancer chemopreventive activity (48). In one study samples tested for genetic abnormalities, with deletion of 17p., and exhibiting resistance to F-ara-A ($IC_{50} > 10 \mu M$), consistent with the crucial effect of p53 on sensitivity to fludarabine remained sensitive to PEITC. The loss of p53 is known to promote genetic instability and mitochondrial dysfunction (49), which not only confers drug resistance but may also promote ROS production (50). In this way it is conceivable that the p53-null CLL cells may have elevated ROS generation and would be highly sensitive to PEITC. Since the loss of p53 is prevalent in cancer and associated with resistance to many standard therapeutic agents (51), the novel ROS-mediated strategy using agents such as PEITC may have potentially broad clinical implications. The increase in ROS generation in CLL cells may render them highly sensitive to PEITC, whereas normal lymphocytes with low ROS output are less vulnerable to this compound. CLL cells from patients in advanced stages refractory to fludarabine-based therapy still remain highly sensitive to PEITC due to their increased ROS generation (52).

To improve their efficacy, HDACi have been paired with other antitumor agents. There are several combination therapies, such as ROS-generating agents, that together may provide a therapeutic advantage over single-agent vorinostat (53).

This last combination of Vorinostat and a ROS generating agent like, Phenylethyl-isothiocyanate (PEITC), has already been tested by us in nanoparticles systems with very

good results, and so, it would be a best to associate them in this way for the production of the next generation of BNPs.

7.3 Histone deacetylase inhibitors (HDACi)

Histone acetylation is another recent alternative for CLL treatment that consists in a posttranslational modification that plays a role in regulating gene expression.

More recently, other non-histone proteins have been identified to be acetylated which can regulate their function, stability, localization, or interaction with other molecules. Modulating acetylation with histone deacetylase inhibitors (HDACi) has been validated to have anticancer effects in preclinical and clinical cancer models. This has led to development and approval of the first HDACi, Vorinostat, for the treatment of cutaneous T cell lymphoma. The impressive anticancer activity observed in both in vitro and in vivo cancer models, together with their preferential effect on cancer cells, have led to a huge effort into the identification and development of HDACi with different characteristics. To date, several clinical trials of HDACi conducted in solid tumors and hematological malignancies have shown a preferential clinical efficacy of these drugs in hematological malignancies and in particular in cutaneous T-cell lymphoma (CTCL), peripheral T-cell lymphoma (PTCL), Hodgkin lymphoma (HL) and myeloid malignancies. Several agents are also beginning to be tested in combination therapies, either as chemo-sensitizing agents in association with standard chemotherapy drugs or in combination with DNA methyltransferase inhibitors (DNMTi) in the context of the so-called "epigenetic therapies", aimed to revert epigenetic alterations found in cancer cells. Vorinostat has demonstrated also efficacy in Hodgkin and Non-Hodgkin Lymphomas (53-56). However, to date, targeting acetylation with HDACi as a monotherapy has shown modest activity against other cancers including B-CLL.

7.4 New biodegradable nanoparticle systems for the treatment of B-chronic lymphocytic leukemia and lymphomas

The pharmaceutical industry as well as the scientific community has been dedicating a big effort as well as a large financial investment in the generation of promising targeted new therapeutic approaches for the treatment of all types of cancers including B-CLL and other Non-Hodgkin lymphomas. These attempts have been design mainly to overcome the challenges associated with the development of drug resistance. Also novel strategies are urgently needed to by-pass the adverse side effects of standard and new chemo and biologic therapeutic agents given by oral, IV or IM routes. In this way, we and others, have proposed to use monoclonal coated and drug loaded biodegradable nanoparticles (BNPs) specially to promote cell epitope specific delivery, and an increased sensitivity to anti-neoplastic drugs. Nanoparticle-based systems are already a real possibility to treat B-CLL. This innovative strategy will generate a pipeline of products in the near future, which will change the way we will treat these patients as well as others with different but also complex and severe diseases. There will probably be no big differences between products of this kind but this little varieties will also not be trivial. The best available products such as those non-traditional pro-apoptotic compounds or its combinations, will be the substances to be selected and loaded in these particles, and this will be one of the clues for these particles to work. HCQ is one of the best candidates as seen in all our research studies. It will be important for the hematologist to completely understand the mechanisms of action of this

substance when delivered inside BNPs. Many drugs as well as other biologic agents have poor cellular bioavailability (56). At low or suboptimal concentrations these compounds are not pro-apoptotic at all or their effects could be very different to those we are looking for. Consistent with this, HCQ in clinical trials for B-CLL given orally like for malaria or rheumatic disease, might not be provoking the desired effects or even some, that could be not good at all for the patients, specially considering that its apoptotic effects are only reach at very high concentrations that are impossible to obtained by the oral route. Combining HCQ with CLB will probably be synergistic especially for those patients with already resistant disease or with bad prognosis gene mutations (57). Also PEITC associated with Vorinostat as well as Parthenolide with Honokiol/Magnolol seem to be interesting combinations to try. In relation to the monoclonal antibodies that will coat the first generation of BNPs for B-CLL treatment, those that target the anti-CD20 receptor alone or in combination with the anti-CD19 antibody are surely the best candidates to begin. Then the anti-CD52 and anti-CD23 would also be good possibilities to be used. There are some biological needs for these treatments to succeed: (i) they must exhibit "stealth" qualities to evade macrophage attack and the immune response; (ii) be nontoxic, traceable and biodegradable following systemic administration through any route; (iii) display effective pharmacokinetic properties; (iv) the polymer must protect the embedded therapeutics; and (v) they must be selective in their targeting to specific tissue sites. All these qualities are fulfilled by our BNPs. Regulatory changes are needed also at the FDA. FDA must provide clear regulatory/safety guidelines for therapeutic nanoparticles including those related to environmental and health issues, but must help to accelerate the introduction of these technologies as soon as possible in the market.

8. Production and characterization of the first generation of biodegradable nanoparticles and its use in the treatment of B-CLL

We have already described that almost all FDA approved antineoplastic drugs in clinical use today are not selective to cancer cells and can produce very toxic side effects (58). In order to obtain better results and tolerability other therapeutic strategies should be developed, specially those that could carry drugs in new delivery systems designed to specifically target cell receptors or epitopes an introduce the desired therapeutic agents loaded in a carrier such as a nanoparticle. These systems are a new technology for cancer therapy (59). Receptor-targeted nanoparticles like the ones presented here (200~300 nm) are good drug carriers and can transport large amounts of drugs, while having a prolonged circulation time (specially when surface PEGylated), as well as a very selective tumor penetration when coated with monoclonal antibodies such as the anti-CD20 Rituximab. These nanoparticles can release enough amounts of drugs inside the cancer cells and in this way overcome multidrug resistance (MDR) mechanisms which are over-expressed in many B-CLL cells (60). Other nanoparticles (e.g., liposomes, micelles, polymers, dendrimers) have demonstrated efficacy both in vitro and in vivo (61). Nanoparticles systems have emerged as important tools to modify the release profile for a large number of drugs including inhibitors, protein and peptide molecules. They are produced from biocompatible and biodegradable FDA approved materials, making them a promising therapeutic strategy for drug targeting and delivery, and surmounting the inherent limitations of regulation acceptance. Additional advantages include reduction of drug toxicity and increase of drug bioavailability . Several previous studies have already demonstrated the goal, and it is well

known that when used to deliver chemotherapeutics to cancer models, nanoparticles have a higher maximum tolerated dose than free drug. Nanoparticles for example provide a promising carrier for cisplatin administration avoiding its side effects without a reduction of the efficacy, which was consistent with a higher activation of apoptosis than free-drug. Moreover, this simple strategy can promote co-assembly of drugs, imaging agents and targeting moieties into multifunctional nano-pharmaceutics. Most current anticancer agents do not greatly differentiate between cancerous and normal cells, leading to systemic toxicity and adverse effects. This lack of differentiation greatly limits the maximum drug allowable dose, but the overexpression of receptors or antigens in human cancers lends itself to efficient uptake by receptor mediated endocytosis. There is some previous but limited published experience with nanoparticles in lymphomas, including *in vivo* studies in lympho-proliferative diseases. We have already published (3) the possibility of using these pharmaceutical new systems to overcome drug resistance of B-Chronic Lymphocytic Leukemia cells. We used for this purpose, PEG-PLGA polymers based BNPs specially designed to be loaded with HCQ and coated with specific monoclonal antibodies. These BNPs induced apoptosis of malignant B-CLL cells at low concentrations. HCQ-BNPs with mAbs induced a decrease in cell viability in a dose and time-dependent manner. In leukemic cells, the nanoparticles reduced cell viability in doses and times significantly lower than in normal lymphocytes. *In vitro* treatment of drug-resistant B-CLL cells with these HCQ-loaded BNPs was shown to be significantly more effective ($P < 0.001$) than BNPs without drug, indicating that treatment with empty BNPs had little impact on cell viability. BNPs encapsulated with HCQ, but without mAbs, had significantly less impact on *in vitro* cell viability ($P < 0.001$). Anti-CD19 and anti-CD20 antibodies suspended in PBS with 10% BSA in AIM V medium alone without BNPs produced no significant apoptotic effect in B-CLL or normal lymphocytes. Active targeting is based on specific interactions with receptors on target cells that may promote enhanced internalization of nanoparticles through receptor mediated endocytosis. In addition, a common method for reducing the recognition of nanoparticles by the RES is to coat their surfaces with polyethylene glycol (PEG). In addition to specific interactions between the ligands on the surface of nanoparticles and receptors expressed on the tumor cells, this may trigger receptor mediated endocytosis. Furthermore, active targeting has shown the potential to suppress multidrug resistance (MDR) via bypassing of P-glycoprotein-mediated drug efflux. The targeting ligands may not play a role until the targeted nanoparticles find the tumor sites. This is very easy for our particles as the targeted cells reside mainly inside the vascular compartment or in tissues with high accessibility to the vasculature, such as in the case of B-CLL. In this situation, targeting occurs relatively quickly and easily. When BNPs were coated with human anti-CD19 and anti-CD20 antibodies the apoptotic effect was more pronounced and enhanced. Confirming the idea that nano-constructs such as these ones targeting B-CLL cells should serve as customizable, targeted drug delivery vehicles capable of ferrying large doses of chemotherapeutic agents into malignant cells while sparing healthy ones. Our nanoparticles beside the original idea of loading them with an anti-malarial immune modifying drug such as HCQ, have the advantage of their special production process in which nano core shells of a median diameter of 250 nm can be obtained by a non-emulsion-polymerization method, and in which several different drugs or peptides can be easily encapsulated while one or more monoclonal antibodies can be coated in their surface. We also tried a similar kind of nanoparticles, both, *in-vitro* and *in-vivo*, in a mice living model of human lymphoma, but

HCQ was combined this time with CLB in order to potentiate its effects, and Rituximab, the first anti-human CD20 monoclonal antibody approved by the FDA for the treatment of lymphomas (62), was attached to the surface of the BNPs. We have developed a special type of biodegradable targeted nanoparticles with demonstrated efficacy *in vitro* and *in vivo* in animal studies for B-CLL, lymphomas and autoimmune diseases. The rational design of these nanoparticles considered the possibility of delivering high concentrations of HCQ and its combination with CLB loaded in biodegradable (PLGA) nanoparticles and coated with the mentioned antibody. Ordinarily, CLB-resistant B-CLL lymphocytes are 5- to 6-fold more resistant *in vitro*, using the MTT assay, as compared to sensitive lymphocytes (IC₅₀ CLB of ≈ 61.0 mmol=L can be considered sensitive). This is why delivering CLB in nanoparticle systems seems to be a good idea indeed in resistant phenotypes. Loss of viability in human CLL cells correlated with the early induction of apoptosis.

9. Encapsulation of CLB and HCQ sulfate in BNPs containing anti-human-CD20 monoclonal antibody rituximab functional groups on their outer shells

BNPs with an average diameter in the range of 250 nm (measured by Dynamic Light Scattering) were produced by a non-emulsion-polymerization proprietary technology (Bio-Target Inc. USA). The particles had a -0.05 mV surface potential, measure by its zeta potential. The nano- capsules used in this study included a shell region and a core region. The shell was made of three biocompatible biodegradable polymers: PEG-PLA (polyethylene-glycol-poly-lactic-acid) and PCL (polycaprolactone). The monoclonal antibody Rituximab was coated in their surface.

The core included two therapeutic agents: HCQ and CLB. In this way a capsule including two encapsulated anti-neoplastic agents were produced by this methodology. One specific aspect of these BNPs, was an anti human CD20 functional group (Rituximab) dispersed on the outer surface of the shell region. Three different kinds of BNPs were specially designed. BNP0: only polymer-BNPs (PEG-PLA-PCL) at a concentration of 1.66 mg/ml. BNP1: Polymer (PEG-PLA- PCL) at a concentration of 1.66mg/ml coated with the anti human-CD20 monoclonal antibody, Rituximab, at a concentration of 8.824 ug/ml, combined with a cyanine 5.5 (CY5.5) dye, a fluorescent molecular beacon that emits photons in the near-IR, at 0.465 ug/ml. BNP2: antihuman- CD20-BNPs + (HCQ + CLB) with polymer (PEGPLA- PCL) at 1.66mg/ml, Rituximab at 8.824 ug/ml, CY5.5 dye at 0.465 ug/ml, and CLB at 5mg/ml-HCQ at 5mg/ml. The particles were produced under class 100 clean room conditions and the CY5.5 dye was chemically attached after preparation of the base BNPs as described below. B-CLL Cells and Cell Culture with BNPs Heparinized blood was obtained after informed consent from 3 B-CLL (median age 64.4 years old) Rai-IV, p-53 mutated, patients. The mononuclear cell fractions were isolated by centrifugation on Ficoll-Hypaque gradients. Primary tumor B cells positive for CD5, CD19, CD23, CD20, CD38 and ZAP70, with unmutated Ig genes and p53 mutations were isolated from the B-CLL patient's mononuclear cell fractions respectively using a B-Cell Isolation Kit. Briefly, 2×10^5 freshly isolated cells, from the CLL patients, were incubated in triplicate for all experiments at 37°C and 5% CO₂ at various concentrations of BNPs in RPMI 1640 serum-free medium. All BNPs were suspended at the time of use in PBS with 10% Bovine Serum Albumin (BSA) at a final total mass concentration of 900 ug/ml. Aliquots of 0.2, 0.5, 1, and 2 ul from these solutions were used for experiments. After 24 and 48 hours of incubation at 37°C, the number of residual

viable cells was estimated in each BNPs system using Ethidium Bromide/Acridine Orange staining and immune-fluorescent microscopy counting as well as measurement of cell apoptosis using annexin-V and propidium-iodide by FACS analysis. Direct Tumor Cell Cytotoxicity Assays using BJAB and MEC-1 cells were also done. The procedure was modified from Macor et al. (63) in order to evaluate the effect of BNPs on BJAB cells, a human Burkitts lymphoma cell line already used to characterize Rituximab activity and on MEC-1, a cell line derived from a patient with chronic lymphocytic leukemia. 2×10^5 cells of each class were incubated in triplicate in RPMI 1640 serum-free medium (Sigma-Aldrich Italy) with various amounts of BNPs. All BNPs were suspended at the time of use in PBS with 10% Bovine Serum Albumin (BSA) at a final total mass concentration of 900 $\mu\text{g}/\text{ml}$. Aliquots of 0.2, 0.5, 1, and 2 μl from these solutions were used for experiments. After 48 hours of incubation at 37°C , the number of residual viable cells was estimated using the MTT assay and percentage of dead cells was calculated. For toxicology Studies, 4 groups of 4 C57/Bl mice each were treated intraperitoneally with different amounts of BNP2 in order to evaluate their effects on healthy animals: Group 1 received 10 μl of BNP2 for 4 times (50 μg HCQ + 50 μg CLB). Group 2 received 20 μl BNP2 for 4 times (100 μg HCQ + 100 μg CLB) and Group 3 received 40 μl BNP2 for 4 times (200 μg HCQ + 200 μg CLB). Group 4: 80 μl BNP2 for 4 times (400 μg HCQ + 400 μg CLB). Each animal was treated on days 0, 2, 4 and 7 and followed up to day 21. Therapeutic studies using a Human/Mouse Model of Burkitt's Lymphoma were performed. Female severe combined immunodeficiency (SCID) mice (4-6 weeks of age) were purchased from Charles River and maintained under pathogen-free conditions. A SCID xenograft model of Human Lymphoma specially developed to investigate the *in vivo* distribution and therapeutic effects of monoclonal antibodies was used to analyze the effects of BNPs after the toxicology studies. BJAB cells were expanded *in vitro* and then implanted intraperitoneally (2×10^6 cells/mouse) in 15 SCID mice (day 0). Ten of these mice were used as controls and the other 5 mice were treated *i.p.* with 80 μl BNP2 (400 μg HCQ + 400 μg CLB) at days 4, 7, 10, 13. We analyzed survival of controls and treated mice for more than 120 days (end of experiment). Histological and immunohistochemicals analysis were done. Tumor peritoneal masses and liver were collected from sacrificed mice (for ethic reasons) and maintained in buffered-formaline for 16 hours. Samples were first washed in EtOH 70% for 2 hours and then in EtOH 100%. Histological and immunohistochemicals analysis were performed. Results obtained from the prior assays shown that BNPs2 formulated with the human anti-CD20 monoclonal antibody Rituximab, and drugs (HCQ and CLB) efficiently induced apoptosis of malignant human B-CLL cells *in vitro*. At 0.5 μl concentration, and 24 hs of culture, these BNPs2 induced 4-fold more apoptosis in malignant B-CLL cells compared to BNPs0 and BNPs1, reaching at 48 hs of culture almost a 95% cell killing effect. Reductions of living B-CLL cells were observed *in vitro* at 24 and 48 hours for injections of all concentrations of BNPs2. Loss of viability correlated with early induction of apoptosis as confirmed by monitoring the B-CLL cells after Annexin V/propidium iodide staining. BNPs2 with Rituximab and drugs induced a decrease in cell viability in a dose and time dependent manner. *In vitro* treatment of these B-CLL cells with BNPs2 showed to be more effective than BNPs without drugs (BNP0) indicating that treatment with empty BNPs had little impact on cell viability. BNPs1 encapsulated with HCQ and CLB but without monoclonal antibody had almost no impact on *in vitro* cell viability. The killing effect of the different BNPs on the B-CLL derived cell line MEC-1 was also analysed. BNPs0 and BNPs1 had 0% cell killing effect after 48 hs

in culture, while BNPs2 had 94% killing effect using 0,5 ul. One or 2ul concentrations added little killing effect in the assays. The same experiments performed with BNPs and the Burkitt's lymphoma cell line (BJAB) showed similar results, obtaining levels of 94% cell killing with 1 ul of BNPs2. In vivo studies start analyzing BNP2 toxicity in healthy mice. C57/Bl mice were treated intraperitoneally with different amounts of BNP2 (10 ul, 20 ul, 40 ul or 80 ul) for 4 injection on days 0, 2, 4 and 7 and followed up to day 21 in order to see possible adverse effects. No side effects have been detected in all the study period in any of the animals injected with BNPs. Histological and immunochemical studies performed on survival animals do not produce information about the distribution of the BNPs because the analysis was performed several weeks after BNPs injections. The in vivo therapeutic effect study was concluded 120 days after the administration of the tumor cells to the animals. Control mice died within day 63 after tumor cell injection. Three treated mice died between day 72 and 98 and tumor mass and liver were collect from these animals. At day 120 only 2 treated mice appeared healthy even at the end of the study. Histological studies were performed on samples derived from tumor masses developed in the peritoneum of treated and untreated mice. The peritoneal masses observed in all untreated animals were mainly composed of sheets of homogeneous round- shaped, medium-sized malignant lymphoid elements showing cohesive growth. However, necrotic areas were seen in peritoneal histological masses derived from all BNPs2-treated mice.

10. Final discussion

We have studied and described a new kind of therapeutic BNPs with a functional specific group attached to their outer shell, the first human anti-CD20, FDA approved for Lymphoma treatment, monoclonal antibody Rituximab. We have also combined in these BNPs an antimalarial agent, Hydroxychloroquine, known to have pro-apoptotic properties, with the old anti-leukemic drug Chlorambucil. This last agent has been the gold standard treatment for B- CLL for many decades until the general acceptance of Fludarabine as first line choice treatment (64). Both drugs have been used alone or in combination with Rituximab and other agents for the treatment of many B cell malignancies (65). After initial good responses, these hematological neoplasias usually mutate, and become resistant to all modalities of standard treatments. B-CLL and Lymphomas seem to be different sides of the same coin. Their malignant cells have the same potential to kill the patients, but also the same potential to dye by apoptosis under similar targeted therapies, like the one we propose here. With the use of these BNPs we were able to specifically target a variety of B malignant cells such as those from B-CLL patients, as well as BJAB and MEC-1 cell lines, with outstanding cell killing efficiency by apoptotic mechanisms. These BNPs induced high levels of responses beside having some of those cells, like the ones from CLL patients, bad prognostic markers such as mutation of the p- 53 gen. Then, a BNP coated with Rituximab and loaded with HCQ and CLB could be an interesting therapeutic strategy in which the antimalarial drug with pro-apoptotic activity seems to have a synergistic effect when associated with a cytotoxic agent. Those mechanisms of drug resistance usually found in Lymphomas after several treatment modalities could be overcome by the use of these BNPs and this drug combination. We did not see any adverse effect related to the use of BNPs when tested in living mice models. This could be a good evidence of the safety of this kind of treatment. The survival advantage of those animals implanted with human lymphoma cells when treated with BNPs is provocative, but in some way it was expected after the good

results obtained in our in vitro assays. This prolonged overall survival of the treated animals probably correlates well with some of the histological findings, in which cell apoptosis and necrosis were seen only in B cell tumor areas after injecting the mice models with BNP2. For all of this, it seems reasonable to do more animal studies of the same kind in order to accelerate a possible introduction of this promising technology into a first human clinical trial. Maybe changing at last, the high mortality associated with B-CLL and other indolent lymphomas.

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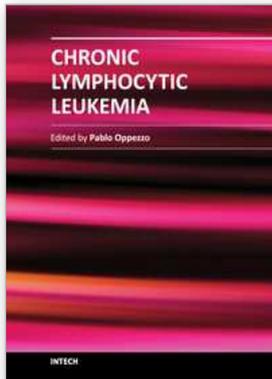
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B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancytopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL's cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
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Phone: +86-21-62489820
Fax: +86-21-62489821