**Abstract**

Apoptosis inhibition has been demonstrated as a characteristic of the Non-Hodgkin’s Lymphoma (NHL). The up-regulation of anti-apoptotic BCL-2 family proteins allows the survival of cancer cells. Therefore, the strategy to induce apoptosis by targeting BCL-2 proteins is the basis to propose new therapeutic probes for NHL. The aim of this research was to prepare a multimeric and multivalent system of gold nanoparticles (20 nm) conjugated to Anti-CD20 and to evaluate its biological potential to decrease the anti-apoptotic BCL-2 protein levels. The Anti-CD20 monoclonal antibody was conjugated to gold nanoparticles (AuNP) by means of spontaneous reaction of the thiol groups of cysteine. To obtain AuNP-AntiCD20, the AntiCD20 was added to 1.0 mL of AuNP solution (1 nM) at 18°C with stirring for 10 min. The nanosystem was characterized by TEM, DLS, FT-IR and UV-Vis. In vitro studies were carried out in lymphoma cells. The BCL-2 protein concentration was quantified by enzymatic immune assay after treatment with AuNP or AuNP-AntiCD20 or AntiCD20 (positive control). TEM and spectroscopic techniques demonstrated that AuNPs were functionalized with AntiCD20. Raji cells treated with AuNP-AntiCD20 showed the lower BCL-2 concentration (13.07 ± 0.02ng/mL) with respect to that of incubated with the non-functionalized AuNP (14.45 ± 0.31ng/mL) or AntiCD20 (19.85 ± 0.63ng/mL), indicating that the multimeric and multivalent AntiCD20 nanosystem enhanced the BCL-2 concentration reduction with the possible apoptosis increasing. AuNP-AntiCD20 demonstrates properties suitable for using as a target-specific agent useful for the NHL treatment.

**Keywords:** Non-Hodgkin’s Lymphoma; Rituximab; Gold Nanoparticles; BCL-2

**Introduction**

Programmed cell death (apoptosis) avoidance has been demonstrated as a characteristic of Non-Hodgkin’s Lymphoma (NHL) and it is intensively studied in order to develop new therapeutic approaches. The up-regulation of anti-apoptotic BCL-2 family proteins (BCL-2, BCLXL, BCLw, MCL1, BCLB and BFL1), allows the survival of cancer cells by means of preventing mitochondrial outer membrane permeabilization. The strategy to induce apoptosis by targeting BCL-2 family proteins is the base to propose a new therapeutic approach for NHL [1].

Around 80–85% of NHL are B-cell malignancies and more than 95% of these express surface CD20 antigen which is not found on stem cells, pro-B cells, normal plasma cells or other normal tissues [2-4]. AntiCD20, a chimeric monoclonal antibody approved for the FDA for the treatment of refractory or relapsed B-cell lymphoma binds specifically to the CD20 antigen. AntiCD20 binds complement and induces antibody-dependent cellular cytotoxicity against malignant B cells, also inhibits cell proliferation and induces apoptosis [3-5].

The clinical effect of antiCD20 monoclonal antibody treatment alone is considered still unsatisfactory unless very high doses are used [6-8]. The cells, in which an excess of BCL-2 is produced, are inadequately long-lived even with endogenous or exogenous death stimuli. The elevated levels of BCL2 found in many lymphoid malignancies can contribute to therapy resistance by blocking apoptosis [9].

Recent studies have confirmed that conjugating peptides to gold nanoparticles (AuNP) produces biocompatible and stable multimeric and multivalent systems with target-specific molecular recognition. The bio-molecules can be conjugated to gold nanoparticle surface through the spontaneous reaction of the AuNP with a thiol (cysteine) or an N-terminal primary amine [10-16]. It has been demonstrated that rituximab (antiCD20) can be successfully attached to the surface of gold nanoparticles (30 nm), creating multivalent antibody complexes that selectively bind to the cell membrane of CD20 positive cells [17,18].

We have hypothesized that the treatment of lymphoma cells with the AuNP-AntiCD20 nanoconjugate, would efficiently reduce BCL-2 levels with the possible apoptosis induction because of the multimeric and multivalent interactions of AuNP-AntiCD20 and the synergic effect between AuNP and AntiCD20 antibody.
The aim of this research was to prepare a multivalent system of Anti-CD20 functionalized to gold nanoparticles (20 nm) and evaluate its biological potential to decrease the anti-apoptotic BCL-2 protein levels.

**Experimental Procedures**

**Materials**

Rituximab (MabThera, 10 mg/mL) was purchased from Roche Inc. Trisodium citrate dehydrate and tetrachloroaquoric acid were purchased from Sigma-Aldrich Company and used as received.

**Methods**

**Synthesis of gold nanoparticles (AuNP suspension):**

Previously we reported the synthesis of AuNP obtained in type I water or injectable-grade water ([14]. Briefly, an aqueous solution (50 mL, Type I water) of 1.7mM (to produce AuNP with average diameter of 20 nm) trisodium citrate dehydrate (Sigma-Aldrich) was brought to boiling condition and stirred continuously, then 1 mL of 1% tetrachloroauric acid (HAuCl₄·3H₂O, Sigma-Aldrich) was added quickly at one time, resulting in a change in solution colour from pale yellow to black to deep red. AuNP solution was dialyzed against injectable-grade water (5 changes of 0.5 L) during 3 h and sterilized by membrane filtration (Millipore, 0.22 µm).

**Preparation of AuNP-AntiCD20 multimeric system:**

Coating the nanoparticles by chimeric Anti-CD20 monoclonal antibody was obtained by mixing 10 µL of Anti-CD20 (6 x 10¹² molecules; 1µM in type grade water) with 1 mL of the AuNP solution (1 nM, 6.58 x 10¹¹AuNP/mL) therefore, 10 Anti-CD20 molecules per nanoparticle were attached. The mixture was stirred for 5 min in order to allow the complete exchange of citrate with thiol on the particle surface. No purification was required.

**TEM:**

The AuNP and AuNP-AntiCD20 system were characterized in size and shape by TEM using a JEOL JEM 2010 HT microscope operated at 200 kV. The samples were prepared for analysis by evaporating a drop of the aqueous product onto a carbon-coated TEM copper grid.

**UV–Vis spectroscopy:** Absorption spectra in the range of 400–800 were obtained with a Perkin Elmer Lambda-Bio spectrometer using 1-cm quartz cuvette. AuNP and AuNP-AntiCD20 were measured by UV–Vis analysis to monitor the AuNP surface plasmon resonance band.

**Particle size and zeta potential:** The zeta potential and particle size range (in suspension) of AuNP and AU-CD20 was measured (n = 3) using the particle size (dynamic light scattering = DLS) and Z potential Nanotrac-analyzer (Nanotrac Wave, Model MN401, Microtrac, FL, USA). The measurement of Z-potential is based on the velocity and direction of nanoparticles exposed to an electric field and the particle size range including its polydispersity was determined based on measuring the time dependent fluctuation of scattering of laser light by the nanoparticles undergoing Brownian motion.

**FT-IR:** Fourier transforms infrared (FT-IR) spectroscopy of AuNP and AuNP-AntiCD20 were acquired on a Perkin Elmer System spectrometer (Spectrum 400) with an ATR platform (Diamond GLADIATOR, Pike Technologies) using attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy from 100 to 700 cm⁻¹ (Far infrared) or 500-4400 cm⁻¹ (Mid Infrared).

**Sulphydryl motifs quantitation:** Sulphydryl groups per molecule were calculated using an assay with Ellman’s reagent (5,5' -dithio bis-(2-nitrobenzoic acid)); 50 µl of a 0.01M solution of Ellman’s reagent and 2.5 mL of 0.1 M phosphate buffer with 1 mM EDTA at pH = 8 were added to the samples. Then 250 µL of the AuNP-AntiCD20 were added. The reaction was mixed and left at room temperature for 15 min. Absorbance measurements were made at 412 nm. The sulphydryl groups were calculated with the molar extinction coefficient of Ellman’s reagent and the concentration of antibody added to each nanoconjugate.

**BCL-2 quantitation:** The effect of AuNP-CD20 nanoconjugate on the expression of BCL-2 protein concentration was evaluated using the Human BCL-2 Platinum Elisa Kit (eBioscience, San Diego,USA). The kit is an enzyme-linked immunosorbent assay for the quantitative detection of human BCL-2.

The human CD20 positive cells (Raji cell line), approximately 2.5 x 10⁶ cells/mL were treated with AuNP (n=5) or AuNP-AntiCD20 (n=5) or Anti-CD20 (n=5). The concentration of the Anti-CD20 solution (positive control) was 10 mg/mL. The solution was mixed with culture medium for the treatment of the cells at a final concentration of 2.5 mg/mL.

**Statistical analysis:** The difference between AuNP and AuNP-AntiCD20 BCL-2 concentration was evaluated with the Student’s t-test (significance was defined as P <0.05).
Results and Discussion

Size and distribution

The characteristic surface plasmon resonance (SPR) band of gold nanoparticles was observed at 519 nm in the UV-Visible spectrum (Figure 1a), confirming the presence of spherical and monodispersed gold nanoparticles (Figure 1b). TEM micrographs showed spherical gold nanoparticles and a diameter of 21.04 ± 2.19 nm determined by DLS (Figure 1c).

In the case of AuNP-Anti-CD20, the UV-Vis spectra (Figure 2a) showed a characteristic shift of the surface plasmon resonance band (524 nm), with respect to that of AuNP (519 nm). The shift in the surface plasmon resonance position occurs as a result of the antibody adsorption on the AuNP surface, additionally no significant aggregation was observed (Figure 3). It was possible to observe a ‘corona’ around the gold nanoparticle due to the poor interaction of the electron beam with the Anti-CD20 molecules (low electron density), in contrast with the strong scattering of the electron beam when it interacted with the metallic nanoparticles. The hydrodynamic diameter measured by DLS of the nanoconjugate dispersed in solution, was 169 ± 10.1 nm (Figure 2a and 2b).

Zeta potential

A Z-potential of -43.8 ± 3.54 mV was obtained for the multimeric system AuNP-Anti-CD20. This result provides a magnitude of the repulsion or electrostatic attractions between the functionalized nanoparticles indicating the formation of a stable colloid.

FT-IR

The main bands and the assignation of the ATR-FTIR AuNP, Anti-CD20 and AuNP-AntiCD20 spectra of the lyophilized samples are shown in Table 1. From the IR spectra it is also clear that the AuNP displaces the carboxylate groups of the citrate and significantly interacts with the AuNP because of the presence of intermolecular and intramolecular hydrogen bonding (several bands from 700 to 350 cm⁻¹). As it was expected, the Au-S bond was observed at 287 cm⁻¹ in the AuNP-Anti-CD20 spectrum (Figure 4).

Sulfhydryl group quantitation

The calculated sulphydryl groups per Anti-CD20 molecule were 13.8 ± 5.8. Theoretically each Anti-CD20 molecule has 32 sulphydryl groups, hence with this results it is assumed that approximately 19 cysteine residues are attached either to the gold nanoparticle of forming disulfide bridges.

BCL-2 quantitation

The ELISA assay demonstrated a BCL-2 concentration of 13.07 ± 0.02 ng/mL for the multimeric system AuNP-AntiCD20, which was significantly lower (P< 0.05) compared with that of AuNP (14.45 ± 0.32 ng/mL) or AntiCD-20 (14.45 ± 0.32 ng/mL) (Figure 5).

This methodology provides consistent evidence that BCL-2...
In vitro Decrease of the BCL-2 Protein Levels in Lymphoma Cells Induced by Gold Nanoparticles and Gold Nanoparticles-Anti-CD20

Table 1: Main IR vibrational frequencies (cm⁻¹) for AuNP, Anti-CD20 and AuNP-Anti-CD20.

<table>
<thead>
<tr>
<th>Functional group/vibrations</th>
<th>AuNP</th>
<th>AntiCD20</th>
<th>AuNP-AntiCD20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl (-OH)</td>
<td>3308</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amide I ν (N-H)</td>
<td>-</td>
<td>3267</td>
<td>3307</td>
</tr>
<tr>
<td>Amide overtone II (N-H)</td>
<td>-</td>
<td>3074</td>
<td>3168</td>
</tr>
<tr>
<td>ν₁ (-CH₃); ν₂ (-CH₂)</td>
<td>2880, 2935</td>
<td>2962, 2934</td>
<td>2940, 2884</td>
</tr>
<tr>
<td>Amide I ν(C=O)</td>
<td>-</td>
<td>1634</td>
<td>1646</td>
</tr>
<tr>
<td>Amide II (N-H)</td>
<td>-</td>
<td>1565</td>
<td>1575</td>
</tr>
<tr>
<td>Amide III ν(C-N)</td>
<td>-</td>
<td>-</td>
<td>1436</td>
</tr>
<tr>
<td>Carboxilate ν(COO⁻), νas (COO⁻)</td>
<td>1393, 1586</td>
<td>1391</td>
<td>-</td>
</tr>
<tr>
<td>v(C=C)</td>
<td>1109</td>
<td>1243</td>
<td>1260</td>
</tr>
<tr>
<td>Phenyl ν(C-H); δ(C-H) (Phenylalanine)</td>
<td>-</td>
<td>1077</td>
<td>1040</td>
</tr>
<tr>
<td>Amide I δₚ(N-H)</td>
<td>-</td>
<td>645</td>
<td>664</td>
</tr>
<tr>
<td>Disulphide (S-S)</td>
<td>-</td>
<td>542</td>
<td>529</td>
</tr>
<tr>
<td>C-S-Au (S-Au)</td>
<td>-</td>
<td>-</td>
<td>287</td>
</tr>
</tbody>
</table>

protein is reduced in the Raji Burkitt’s lymphoma cell line exposed to different treatments. Because of the functional importance of BCL-2 in apoptosis control, efforts have been performed to study the role of BCL-2 for prognosis or response to therapy in many tumor diseases. In high-grade B-cell lymphomas, the expression of BCL-2 was associated with poor prognosis. It has been suggested that the observation of direct induction of apoptosis by cross-linked Anti-CD20 might provide additional efficacy to anti-CD20 nanoparticles by the fact that the multiple antibodies per nanoparticle might represent a new efficient method of cross-linking [20].

The non-functionalized gold nanoparticles alone were able to induce reduction in the BCL-2 concentration in CD20 positive Raji Burkitt’s lymphoma cell line (Figure 4). In agreement with this result, Mukherjee et al. observed that non-functionalized gold nanoparticles induced some levels of apoptosis in B-chronic lymphocytic leukemia cells due to down regulation of anti-apoptotic proteins, among them BCL-2 [20-22]. However, we demonstrated that the conjugation of Anti-CD20 to the AuNP enhanced significantly the decrease of BCL-2 levels (Figure 5) with the possible apoptosis induction.

It has been reported that combined therapy between immunotherapy (Anti-CD20, Tositumomab) and radiation (radionuclide, ¹²³I) shows a synergistic effect on the response of BCL-2 in patients with Follicular Lymphoma who have had a relapse after refractor with chemotherapy [20, 23]. A similar synergic effect is expected between the gold nanoparticles and Anti-CD20.

Considering previous publications and the results obtained by evaluating the effect of pro-apoptotic BCL-2 in this research, it can be concluded that AuNP-Anti-CD20 is useful as a multivalent pro-apoptotic agent because of the synergic effect between Anti-CD20 and AuNP. The nanoconjugate is suitable for using as
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Conclusions and Future Directions

In this research it was demonstrated that Anti-CD20 can be successfully attached to the gold nanoparticles surface creating a multimeric and multivalent nanosystem. Characterization methods such as TEM, DLS, UV-Vis, and FT-IR spectroscopy provided evidence of the Anti-CD20 conjugation to gold nanoparticles. This study demonstrates that AuNP-Anti-CD20 could be used to decrease the BCL-2 protein levels with the possible apoptosis induction. However it is necessary to evaluate the AuNP-Anti-CD20 treatment effect by measuring apoptotic Raji Burkitt’s lymphoma cells using flow cytometry.
Acknowledgment

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References