Macedonian pharmaceutical bulletin, **62** (suppl) 537 - 538 (2016)
ISSN 1409 - 8695
UDC: 615.849.2.014.2:615.373
Short communication

Development and standardization of Rituximab-conjugates for labeling with Lutetium-177 and Yttrium-90

Emilija Janevik-Ivanovska[1]*, Darinka Gjorgieva Ackova[1], Katarina Smilkov[1], Icko Gjorgoski[2], Trajce Stafilov[3], Petre Makreski [3], Zorica Arsova-Sarafinovska[4], Lajos Baloch[5], Angela Carollo[6], Alberto Signore[7], Adriano Duatti[8]

[1] *Faculty of Medical Sciences, Department of Pharmacy, Goce Delcev University, Krste Misirkov bb, Stip, Republic of Macedonia*
[2] *Faculty of Natural Sciences and Mathematics, Institute of Biology, University "Ss. Cyril and Methodius" , Arhimedova 3, Skopje, Republic of Macedonia*
[3] *Faculty of Natural Sciences and Mathematics, Institute of Chemistry, University "Ss. Cyril and Methodius", Arhimedova 3, Skopje, Republic of Macedonia*
[4] *Institute of Human Health, 50 Divizija 6, Skopje, Republic of Macedonia*
[5] *Department of Nuclear Medicine and Translational Biological Sciences , National Public Health Center, National Research Directorate for Radiobiology and Radiohygiene, Budapest, Hungary*
[6] *European Institute of Oncology, Division of Nuclear Medicine, via Ripamonti, 435, Milano, Italy*
[7] *Nuclear Medicine Unit, Department of Medical-Surgical Sciences and of Translationa Medicine, "Sapienza" University of Rome, Via di Grottarossa 1035, Italy*
[8] *University of Ferrara, Department of Chemical and Pharmaceutical Sciences, Via L. Borsari, 46 Ferrara, Italy*

Introduction

Our work was focused on the investigation for a ready to use prepared freeze dried rituximab immunoconjugates as potential radiopharmaceuticals for labeling with Lu-177 and Y-90 in order to increase the stability and higher efficiency and lower toxicity. We tested three bifunctional chelating agents (BFCA's), *p*-SCN-Bn-DOTA, *p*-SCN-Bn-DTPA and 1B4M-DTPA conjugated to the same antibody using previously established protocol for conjugation (Gjorgieva Ackova, 2014, 2015; Smilkov, 2014).

The main goal was to investigate chemical characterization of the immunoconjugates, labeled with "cold" non-radioactive isotopes of Lutetium and Yttrium in the same conditions as with radioactive Lutetium 177 and to show the chemical behavior and toxicological properties.

Material and method

The conjugation of antibody with three different bifunctional cleating agents was performed using using previously established protocol for conjugation. The concentrations were adjusted to 1 mg/mL and the solutions were then lyophilized.

The purified immunoconjugates were formulated in absence of any cryoprotectant at the concentration of 10 mg/mL, and subsequently lyophilized according to selected protocols.

The process of freeze drying was completed using Labconco Free Zone Stoppering Tray Dryer, (USA), using protocol described by Park in 2013, modified to our experience.

Concentration of the antibody/immunoconjugate was determinate before and after freeze drying and reconstitution using UV spectrophotometer (Jenway UV/VIS spectrophotometer 6715), and semi-micro UV polypropylene tubes with 0.1M PBS pH=8.0, at 280 nm in triplicate.

After freeze drying both characterization of the conjugates and determination of the average number of BFCA attached to each antibody molecule is performed by MALDI-TOF mass spectrometry and integrity of the antibody was evaluated using SDS-PAGE electrophoresis, on 12% bis-tris acrylamide gel.

The spectroscopic characterization of all three freeze dried immunoconjugates, in terms of monitoring the secondary protein structure (and its preservation), was achieved

* emilija.janevik@ugd.edu.mk

by FT-IR and Raman spectroscopy.

The freeze dying immunoconjugates after reconstitution were labeling with Lu-177 (555 MBq/mg in 0,5 M NH_4OAc) and radiochemical purity was determined by instant thin-layer chromatography on silica plates with a mobile phase of ammonium acetate : methanol (1 : 1) using Cyclone Plus Phosphore Imager (Perkin Elmer).

The obtained radioimmunoconjugates were characterized by SE-HPLC, using a Zorbax Bio Series GF-250 column and the elution process was monitored on UV detector at 280 nm and radiodetector (Wojdowska, 2014).

Toxicological studies were performed in Wistar rats after injection of rituximab labeled with cold Lutetium and Yttrium. Biodistribution studies were performed in 5-6 week old nude mice grafted with Raji cells ($2x10^6$ cells in 0.5 mL medium solution) after injection of radioactive Lu-177-Rituximab.

Results and discussion

The 3 day protocol of freeze drying without the presence of mannitol, showed the greatest similarity in the elution profiles of the immunoconjugate prior lyophilization (Gholipour, 2014).

After freeze drying, the pellets obtained corresponded to the composition and the time until complete reconstitution after addition of saline showed no significant difference in the time of complete dissolution of the lyophilisates, i.e. all tested samples were completely reconstituted in 2 min.

The average number of BFCA attached to each antibody molecule performed by MALDI-TOF mass spectrometry shows presence of two main peaks corresponding to MW of 146491 Da (unconjugated antibody) and 149873 Da (conjugated antibody) which corresponds to an average of 6.1 groups of *p*-SCN-Bn-DOTA (M = 551.61 g·mol-1), two peaks also, corresponding to MW of 146477 Da (unconjugated antibody) and 151246 Da (conjugated antibody) corresponds to an average of 8.8 groups of *p*-SCN-Bn-DTPA (M = 540.54 g·mol-1) attached to a molecule of rituximab and two peaks corresponding to MW of 146848 Da (unconjugated antibody) and 151506 Da (conjugated antibody) corresponding to average of 8.3 groups 1B4M-DTPA (M = 555.58 g·mol-1) attached to a molecule of rituximab.

All immunoconjugates (both before and after lyophilization) were separated in two distinct Mw species which migrated in two bands (upper at ~50 kDa and lower at ~25 kDa) confirming the migration behavior typical for IgG antibodies which are composed of twoidentical subunits each composed by two polypeptide chains: two heavy and two light chains, linked via 4 disulfide bonds. The obtained fragments correspond to molecular masses of rituximab heavy and light chain given at the literature (Bil, 2007;)

In the experimental IR (in the region 2000-500 cm^{-1}) and Raman spectra (2000–400 cm^{-1} region) we observed retaining of native structure of the antibody and no obvious aggregation.

The radiochemical purity and determination of radioimmunoconjugates by SE-HPLC, obtained after radiolabeling the with Lu-177 was higher than 5%. These conjugates were stable for 48h in 0.9% NaCl, however, progressive aggregation was observed.

Animal studies showed no toxicity and SPECT images in mice showed good localization of the tumor, as confirmed by ex-vivo organ counting.

Conclusion

After evaluation of all the obtained results obtained we can conclude:

- Three immunoconjugates were synthesized, using *p*-SCN-Bn-DOTA, *p*-SCN-Bn-DTPA and 1B4M-DTPA using the a selected ratio, 1:20
- Protocol for lyophilization was established, yielding lyophilisates with favorable physicochemical properties.
- The non-radioactive labeling with Y and Lu showed preserved secondary structure in all three types of immunoconjugate, confirming their stability in conditions of freeze-drying and labeling
- During labeling with Lu-177 all three types of radioimmunoconjugates showed high radiochemical purity, over 95%, which was confirmed both in ITLC and SE-HPLC.

The selection of the most appropriate immunoconjugate kit suitable for labeling with Lu-177 or with Y-90 can be made after stability study of the formulation and completition of cell culture studies.

References

Bil, J., Winiarska, M., 2007. Molecular mechanisms of the therapeutic activity of Rituximab. The monoclonal antibody against CD20 antigen. Adv. Cell Biol. 34(2), 335-359.

Gholipour, N., Jalilian, A.R., Khalaj, A., Johari-Daha, F., Yavari, K., Sabzevari, O., Khanchi, A.R., Akhlaghi, M., 2014. Preparation and radiolabeling of a lyophilized (kit) formulation of DOTA-rituximab with 90Y and 111In for domestic radioimmunotherapy and radioscintigraphy of Non-Hodgkin's Lymphoma. DARU J. Pharm. Sci. 22(58), 1-10.

Gjorgieva Ackova, D., Smilkov, K., Janevik-Ivanovska, E., 2014. Formulation and Characterization of "Ready to Use" 1B4M-DTPA-rituximab for Lu-177 Labeling. World J. Med. Sci. 11(4), 535-540.

Gjorgieva Ackova, D., Smilkov, K., Janevik-Ivanovska, E., Stafilov, T., Arsova-Sarafinovska, Z., Makreski, P. 2015. Evaluation of Non-radioactive Lutetium- and Yttrium-labeled Immunoconjugates of Rituximab – A Vibrational Spectroscopy Study. Maced. J. Chem. Chem. Eng. 34, 351–362.

Smilkov, K., Gjorgieva Ackova D., Gjorgovski, I., Janevik-Ivanovska, E., 2014. Rituximab-immunoconjugate kit-formulations for NHL radioimmunotherapy. Physioacta 8(2), 113-120.

Wojdowska, W., Karczmarczyk, U., Maurin, M., Sawicka, A., Laszuk, E., Garnuszek, P., Mikolajczak, R., 2014. In virto and in vivo investigations of 177Lu and 90Y labeled DOTA(SCN)-Rituximab radioimmunoconjugates. Q. J. Nucl. Med. Mol. Imaging 58, 45.