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IN VITRO CITOTOXICITY TESTING OF UBIQUICIDIN 29-41-^{99m}Tc.

Ivette Z. Ocampo[1], Kayo Okazaki[1], Priscila Passos[2], Natalia M. Esteves-Pedro[2], Luis Alberto Pereira Dias[3], Olga Z. Higa[1,2], Fabiana M. da Silva[2] and Daniel P. Vieira[1]

[1] Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP)
Centro de Biotecnologia
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP, Brazil
dpvieira@ipen.br

[2] Laboratório Biosintesis, Ltda.
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP, Brazil
fabiana@biosintesis.com.br

[3] Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP)
Diretoria de Radiofarmácia
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP, Brazil

ABSTRACT

The work carried out cytotoxicity tests using a radiopharmaceutical compound produced at IPEN/CNEN-SP to certify its safety through *in vitro* cytotoxicity tests. Since 2009, the Brazilian regulatory agency (ANVISA) requires that such tests have to be carried out following good laboratory practices (GLP) and in according to the OECD (Organisation for Economic Co-operation and Development) guidelines in order to certify its safety for medical use. Those guidelines comprises series of technical recommendations performed to assure quality of experiments. The study chose Ubiquicidin 29-41, an antimicrobial peptide used to discriminate bacterial infection foci from inflammatory sites. Amounts of UBI_{29-41} were conjugated or not to 99mTc and diluted to equivalent concentrations of 10, 100 and 1000% of the maximum dose (or activity) administered in adults. Possible cytotoxic effects were evaluated in comparison to untreated controls as well as positive and negative damage controls. Both full (radioactive) radiopharmaceuticals, as their precursors (only molecules without conjugation to isotopes) showed no significant cytotoxic effect (citotoxicity $\leq$ 10%). The study was conducted for the first time in the country comprising preclinical testing of this radiopharmaceutical in accordance with internationally accepted quality parameters, ensuring the safety of its use and enabling inclusion in the pharmaceutical regulatory agenda.

1. INTRODUCTION

Nuclear medicine technologies have been used for years to detect or treat various types of pathologies. The use of these technologies often involve the administration of radiopharmaceuticals, which are specific bioactive compounds that carries radioactive isotopes and has the ability to turn cells, tissues or organs into detectable targets. Due to its efficacy and safety, the use of radiopharmaceuticals has been expanded, in general, in all countries. Ubiquicidin is a synthetic cationic peptide with antimicrobial activity with affinity for cell walls of microorganisms [1]. Its use as a diagnostic radiopharmaceutical for fungal infections [2]

and bacterial [3] has been shown to be promising. One of its main positive features is to be able to differentiate septic inflammatory foci regions of aseptic inflammation, helping in the choice of treatment of patients suffering from various infections [4]. This bioactive compound potentially have the ability to induce significant cytotoxic or genotoxic damage, and should be considered as pharmacological non-radioactive compounds; analysis of unlabeled compound is encouraged [5], and thus prone to be tested in pre-clinical trials [6,7,8]. Ubiquicidin is administered in patients in maximum amount of 25µg, (or maximum activity of 10µCi) per injection.

In vitro cytotoxicity tests are performed following well stablished protocols. Usually, Chinese hamster ovary cells (CHO) are exposed to various concentrations of test substances and negative or positive controls. Cell viability can be expressed in percentages relative to unexposed controls. The work tested Ubiquicidin 29-41 (peptide comprising the sequence between aminoacids 29 to 41 from complete protein sequence) conjugated or not with 99m-Tc, an isotope used in nuclear medicine diagnostic procedures, in peptide concentrations carrying up to the equivalent of ten times (10X) maximum injected activity in human adults.

2. MATERIALS AND METHODS

2.1. Cell Cultures

Chinese hamster ovary cells (CHO-KI; ATCC CCL-61) were routinely maintained by Biosintesis Laboratory, Ltda. All experimental cultures were maintained using DMEM as culture medium supplemented with 10% (v/v) fetal bovine serum (FBS) without antibiotics in incubators with constant temperature (37°C) and controlled atmosphere containing 95% O_2 and 5% CO_2. After confluence, cells were detached with Trypsin/EDTA (0.5%/5mM), diluted in complete medium and kept in room temperature until experiments (inferior to 20 min).

2.2. Ubiquicidin 29-41

Commercial Ubiquicidin 29-41 (UBI_{29-41}) was provided as lyophilized powder by the Quality Assurance of Center Radiopharmacy (CR) of the Institute of Nuclear Energy Research (IPEN/CNEN-SP). Using the "standard man" calculations (70kg and 5.5 L of blood), sterile peptide samples were first diluted in deionized water to stock concentrations that were used to make the 0.045 ng/mL of UBI_{29-41} solution in medium (DMEM without serum), corresponding to concentration equivalent to ten times the maximum administered peptide mass to adult patients (10X). Primary dilutions were diluted in DMEM without serum to 0.00045 ng/ml (1/10X) and 0.0045 ng/mL (1X). UBI_{29-41} conjugated with 99m-Tc was produced by Radiopharmacy Center at Nuclear and Energetic Research Institute (IPEN/CNEN-SP), bearing radioactive concentrations from 5 to 6mCi/mL. After arriving at cell culture room, activities were recalculated taking account of elapsed time from production to experimentation using radioactive decay classic equation. Samples were diluted as same as non-radioactive, considering 10µCi as maximum dose in adults. For testing of radioactive peptides, the radioactive concentrations were 0.00018, 0.0018 and 0.018µCi/mL (0.1, 1 and 10X, respectively).

2.3. Cytotoxicity testing

Sodium chloride (0.9% in medium, w/v) was used as negative control (NC), incapable to induce cytotoxicity in the test system. Latex rubber fragments ($1cm^2$) were UV-sterilized (30 min each side in sterile chamber) and added to 10mL of DMEM (0.2g/mL medium). After incubation for 24h (37ºC), latex conditioned media was sterilized by filtering (0.22µm) and used as positive controls (PC), in order to induce strong cytotoxicity in test system. CHO cultures were prepared as suspensions in DMEM+FBS 10%, and plated in 96-well plates in 10,000 cells/well density (100µL).

After allowing 24±2 hours for proper cell adhesion, media were replaced by 100µL of medium containing 0.9% NaCl (negative controls), or 0.5% (v/v) of 2mg/mL latex conditioned medium (positive controls). Experimental wells received 100µL of DMEM containing 0.045 (10X), 0.0045 (1X) or 0.00045 (0.1X) ng/mL. Cells in experiments using 99m-Tc-labelled peptides received 100µL of medium containing 0.018 (10X), 0.0018 (1X) or 0.00018 (0.1X) nCi/mL. Control cells (CC) received 100 µL of medium. All groups were incubated for 24±2 hours in quadruplicates.

Upon completion of this incubation period, culture media was aspirated and cells received 2mg/mL of 3-(4,5-dimetiltiazol-2-il)-5-(3-carboximetoxiphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; *CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay*, Promega) and 0,9% of phenazine methosulfate (PMS, Sigma-Aldrich, CAS 299-11-6) diluted in DMEM (120 µL). After 2 hours of incubation (37ºC, 5%CO_2, 95% O_2), metabolically active cells converted the compound MTS in the presence of PMS in formazan. Absorbance values were obtained by spectrophotometric readings at 490nm, and converted into percentages of cell viability relative to controls (CC). Absorbance values of control untreated cells represented 100% of cell viability. A dilution of test compound was not considered cytotoxic if cell viability values were at least 90% of untreated or negative controls. Two-way ANOVA and Dunnett post-analysis were performed to find statistical relevance between cell viability values comparing to controls.

3. RESULTS AND DISCUSSION

Citotoxicity values obtained from experiments are shown in Fig. 1.

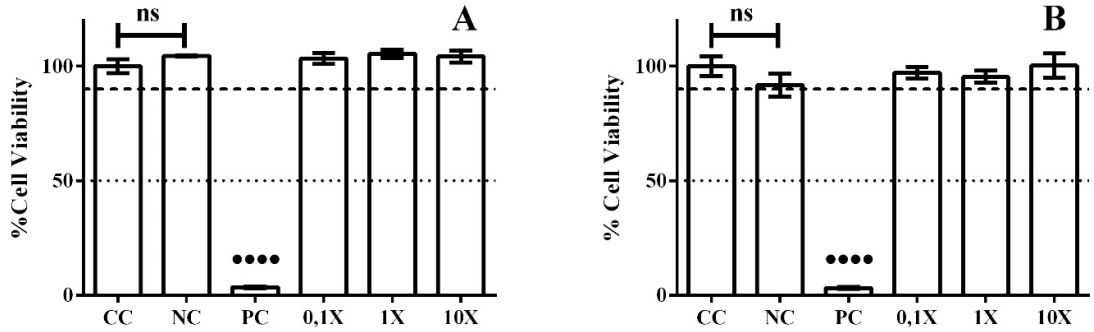

Figure 1: Ubiquicidin 29-41 conjugated (A) or not (B) with 99mTc was not able to induce cytotoxicity in CHO cells. CC: Control cells (only medium); NC: Negative control (NaCl 0.9%); PC: Positive control (latex fragments, 0.5%). 0.1X, 1X and 10X corresponds to 10, 100 or 1000% of maximum activity or concentration allowed to administration in adult patients (10µCi or 25µg). Dashed (----) and punctuated (····) lines indicate 90 and 50% of control cell viability. Bars represent standard error of means. (●●●●): p<0,0001.

The present experiments could not show cytotoxicity in any level in cells exposed to all tested concentrations of ubiquicidin, conjugated or not with 99mTc. Cell viability values of test cultures (% of controls) were not reduced significantly ($p>0.05$). Viability of control (untreated) cells was statistically equivalent to negative controls exposed to NaCl 0.9%. Latex-conditioned medium (PC) induced very prominent cytotoxicity ($p<0.0001$). The work adopted the OECD protocol for cytotoxicity testing (OECD, 2010), which describes any concentration of a given compound as cytotoxic if it can reduce cell viability in at least 10% relative to controls (cell viability equivalent to ≤90%). Regarding this parameter, UBI_{29-41} or UBI_{29-41} 99m-Tc could not be considered cytotoxic.

In this scale, radiation could not induce any damage to cultures. Activities ranging from nanocuries (nCi) per well were not able to raise (or reduce) cytotoxicity, what could lead to an interpretation in which UBI_{29-41} 99m-Tc can be safe even if using radioactive concentrations near from 100µCi/injection when administered in adults. Cytotoxicity of peptide itself also could not be detected by the present test system.

The work could ascertain the safety of ubiquicidin administration using *in vitro* cytotoxicity testing, even if in aberrant and hypothetic case in which a patient could receive ten times the maximum recommended dose.

Side studies are being conducted to assess safety of this peptide regarding genotoxicity.

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REFERENCES

1. Akhtar MS, Qaisar A, Irfanullah J, Iqbal J, Khan B, Jehangir M, Nadeem MA, Khan MA, Afzal MS, Ul-Haq I, Imran MB. "Antimicrobial peptide 99mTc-ubiquicidin 29-41 as human infection-imaging agent: clinical trial." *J Nucl Med*. **46**(4):567-73 (2005)

2. Lupetti A, de Boer MG, Erba P, Campa M, Nibbering PH. "Radiotracers for fungal infection imaging." *Med Mycol*.49 **Suppl 1**:S62-9. (2011).

3. Brouwer CP, Sarda-Mantel L, Meulemans A, Le Guludec D, Welling MM. “The use of technetium-99m radiolabeled human antimicrobial peptides for infection specific imaging.” *Mini Rev Med Chem*. **8**(10):1039-52 (2008).

4. Brouwer CP, Wulferink M, Welling MM. “The pharmacology of radiolabeled cationic antimicrobial peptides.” *J Pharm Sci*. **97** (5):1633-51 (2008).

5. Harapanhalli RS. “Food and Drug Administration requirements for testing and approval of new radiopharmaceuticals.” *Semin Nucl Med*. **40**(5):364-84 (2010).

6. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use - ICH Harmonised Tripartite Guideline. S2 (R1). Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S2_R1/Step4/S2R1_Step4.pdf) (2011)

7. Organisation for Economic Co-operation and Development OECD Guidelines for the testing of Chemicals – Test 487: In vitro Mammalian Cell Micronucleus Test. http://www.oecd-ilibrary.org/docserver/download/9748701e.pdf?expires=1381712350&id=id&accname=guest&checksum=352D2E5C23387C60915480F04D723DE1 (2010)

8. World Health Organization – WHO. Expert Committee on Specifications for Pharmaceutical Preparation - RADIOPHARMACEUTICALS Final text for addition to The International Pharmacopoeia. http://www.who.int/medicines/publications/pharmacopoeia/Radgenmono.pdf. (2008)