Cytotechnology
DOI 10.1007/s10616-016-0024-9

ORIGINAL ARTICLE

In vitro cytotoxic and genotoxic evaluation of peptides used in nuclear medicine (DOTATATE and Ubiquicidin$_{29\text{-}41}$) in CHO-K1 cells

Ivette Zegarra Ocampo · Priscila de Queiroz Souza Passos · Luma Ramirez de Carvalho · Camila Ayala Lira da Cruz · Natália Mencacci Esteves-Pedro · Fabiana Medeiros da Silva · Olga Zazuco Higa · Luiz Alberto Pereira Dias · Kayo Okazaki · Daniel Perez Vieira

Received: 15 March 2016 / Accepted: 24 August 2016

Abstract Micronucleus (MN) assay constitutes a valuable surrogate to the chromosome aberration technique for in vitro testing of the genotoxicity of substances. As test substances, two peptidic compounds (DOTATATE and Ubiquicidin$_{29\text{-}41}$) used in nuclear medicine, were tested for in vitro cytotoxicity and genotoxicity in CHO-K1 cells. None of the compounds showed detectable cytotoxicity (0.5–7.3 ng/mL for DOTATATE and 0.3–4.5 ng/mL for UBI$_{29\text{-}41}$), genotoxicity (0.72, 7.2 and 72.0 ng/ml for DOTATATE and 0.45, 4.5 and 45.0 ng/mL for UBI$_{29\text{-}41}$) or cell cycle changes as compared to untreated controls at the concentrations tested. Statistical analysis showed good concordance between two independent analysts. The results corroborate the notion of the safety of the compounds and present improvements of the in vitro MN assay when performed in a pre-clinical trial context that increase the throughput of small-to-medium testing facilities as an alternative to high content screening systems.

Keywords CHO-K1 cell · Cytotoxicity · Genotoxicity · Modified micronucleus assay · DOTATATE, Ubiquicidin$_{29\text{-}41}$

Introduction

Nuclear medicine technologies have been used since the middle of the last century in order to diagnose and treat patients with oncological diseases (Casar et al. 2016). The use of these technologies often involves the administration of radiopharmaceuticals, which are specific bioactive compounds that carry radioactive isotopes and have the ability to turn cells, tissues or organs into detectable targets.

The success of treatment or diagnosis depends fundamentally upon the choice of an appropriate radionuclide and its carrier molecule, in order to ensure that the radionuclide is delivered directly to the tumor target. Several associations between carriers and isotopes are currently marketed, comprising a family of products for use in human health that shows diagnostic and therapeutic potential (Chaturvedi and Mishra 2016; van Es et al. 2016).

I. Z. Ocampo · L. Ramirez de Carvalho ·
C. A. Lira da Cruz · O. Z. Higa · K. Okazaki ·
D. P. Vieira (✉)
Laboratory of Radiobiology, Center of Biotechnology,
Institute of Nuclear and Energetic Research IPEN/CNEN-SP, Av. LineuPrestes, 2242, São Paulo, SP 05508-000,
Brazil
e-mail: dpvieira@ipen.br

P. de Queiroz Souza Passos · N. M. Esteves-Pedro ·
F. Medeiros da Silva · O. Z. Higa
Biosynthesis Laboratory, São Paulo, SP, Brazil

L. A. P. Dias
Center of Radiopharmacy, Quality Control Management,
Institute of Nuclear and Energetic Research IPEN/CNEN-SP, São Paulo, SP, Brazil

Small peptides present many advantages over other bioactive molecules (like proteins and monoclonal antibodies): they can be easily designed and synthesized to optimize their affinity for a particular receptor and thus display a more specific biodistribution pattern and favorable pharmacokinetics, rapid blood clearance, and can also reach the peptide receptors on tumor cells more efficiently (Okarvi 2008).

[DOTA, Tyr^3] octreotate (DOTATATE) is part of a family of peptidic compounds with affinity for somatostatin receptors (sst2 and sst5) (Johnbeck et al. 2014). Somatostatin receptors are expressed in considerable amounts in cells of neuroendocrine tumors (pancreas, thyroid, colon, breast, gastrointestinal tract) (Nilica et al. 2016) and are associated with intracellular G-protein in the cytoplasm and are internalized after binding with a specific ligand (Cescato et al. 2006). Thus, the use of molecules that mimic the binding of sst is an important tool for the detection and treatment of both primary and metastatic tumors, being widely used for conjugation with the radioactive isotopes (^{177}Lu, ^{131}I, ^{68}Ga) used in positron emission tomography (PET). Another peptide, Ubiquicidin$_{29\text{-}41}$ (UBI$_{29\text{-}41}$) is a synthetic cationic peptide with antimicrobial activity with affinity for the cell walls of microorganisms (Akhtar et al. 2005). Its use as a diagnostic radiopharmaceutical (^{99m}Tc-UBI$_{29\text{-}41}$) for fungal (Lupetti et al. 2011) and bacterial infections has been shown to be promising. One of its main positive features is the ability to differentiate septic inflammatory foci regions from aseptic inflammation, aiding in the choice of treatment of patients suffering from various infections (Ostovar et al. 2013).

For pharmacological safety reasons, it should be considered that these bioactive compounds might also have the ability to induce significant cytotoxic or genotoxic damage and this can be tested with non-radioactive pharmacologically active compounds. Analysis of the unlabeled compounds is encouraged (Harapanhalli 2010) and thus to be preferred for testing in pre-clinical trials. In particular, the genetic toxicity can be evaluated via genotoxicity tests that assess the presence or absence of damage caused directly or indirectly to DNA strands.

Among the various in vitro assays, assessment of the frequency of micronuclei (MN) is one of the methods of choice in the development of toxicological safety tests (OECD 2010). Its performance is based on the count of unrepaired double-strand breaks in the DNA of cells exposed to various damaging agents, chemical or physical, such as ionizing radiation. In interphase cells, the consequences of such breaks are presented as micronuclei, which are small clusters (compared to cell nuclei) of DNA localized apart from, but stained similarly to, the main nucleus. These MN may originate from acentric fragments, as well as from whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division (Fenech 2000). Increased proportions of cells bearing micronuclei (frequency of cells with MN), as well as their quantity in the cytoplasm of analyzed cells (number of MN per cell) are indications of genotoxic damage. This increase may be related to the concentration used of the test substance, leading to an assessment of the genotoxic potential of the same (Speit et al. 2011). The test protocol is performed after exposure of the cells to varying concentrations of a particular test substance. After this period, the cells are fixed, appropriately stained and subjected to analysis by optical (Heddle et al. 2011) or fluorescence (Çelik et al. 2005) microscopy.

High content screening (HCS) approaches are being developed to improve the quality and speed of genotoxicity test results, relying on micronuclei formation (Westerink et al. 2011) or on other types of DNA damage markers (Sobol et al. 2012). These approaches are usually based on the automated analysis of digitally acquired images of binucleated cells with micronuclei, requiring the use of specific equipment. The goal of the present study was to evaluate the cytotoxicity and genotoxicity of non-radioactive DOTATATE and UBI$_{29\text{-}41}$ in CHO-K1 cells through a modified in vitro MN assay, introducing subtle changes in the cell culture and fluorescent staining protocols that offer small-to-medium facilities an intermediate alternative to increase throughput. In this study, non-radioactive DOTATATE and UBI$_{29\text{-}41}$ were tested via non-automated microscopic evaluations. Neither compound showed cytotoxicity or genotoxicity at the concentrations employed.

Materials and methods

Cell line

CHO-K1 cells, subclones of Chinese hamster ovary cells (ATCC CCL-61), were utilized as the test system, following recommendations of the reference for the in vitro testing of chemicals (OECD 2010) and by the fact that these cells present many inherently advantageous characteristics (relatively rapid growth rate with a stable karyotype of 22 ± 2 chromosomes and belong to a genetically stable cell line) (Santos et al. 2014). The cells were maintained in Dulbecco‘s Modified Eagle Medium (DMEM, nº. 12800017, Gibco/Life Technologies, Carlsbad, USA) supplemented with 10 % (v/v) fetal bovine serum (FBS, nº. 12657-029, Gibco/Life Techonologies) without antibiotics in incubators with constant temperature (37 °C) and controlled atmosphere containing 5 % CO_2. After reaching 70–80 % confluence, cells were detached with phosphate buffer saline solution (PBS) + 0.5 % trypsin, and maintained in culture medium for experiments.

Peptides

Commercial Ubiquicidine$_{29\text{-}41}$ (UBI$_{29\text{-}41}$, ABX, Radeberg, Germany) and [DOTA, Tyr3] octreotate (DOTATATE) (PiChem, Graz-Andritz, Austria) were provided as lyophilized powders by the Quality Assurance of the Radiopharmacy Center (CR) of the Institute of Nuclear Energy Research (IPEN/CNEN-SP). For the cytotoxic evaluation, the peptides were prepared in DMEM without serum to obtain concentrations of 0.5–7.3 ng/mL for DOTATATE and 0.3–4.5 ng/mL for UBI$_{29\text{-}41}$. For the genotoxic evaluation, concentrations equivalent to 1/10, 1 and 10 times the maximum dose given to each adult patient were administered to the cells: 0.72 (1/10 times), 7.2 (1 time) and 72 ng/mL (10 times) for DOTATATE and 0.45 (1/10 times), 4.5 (1 time) and 45 ng/mL (10 times) for UBI$_{29\text{-}41}$ Considering the recommended maximum doses for diagnostic purposes, the concentration range of peptides was chosen by considering, as reference, a male of 70 kg body weight with 5.5 L of blood receiving an intravenous injection of 40 µg of DOTATATE or 25 µg of UBI$_{29\text{-}41}$. In this manner, we considered “10 times” the maximum feasible dose administered to each adult patient, simply because the product presentations do not allow higher amounts in a single vial. Cell cultures, cytotoxicity and genotoxicity experiments were carried out following OECD specific guidelines and in accordance to the good laboratory practices (GLP) for regulatory purposes to guarantee safety and efficacy assessment.

Cytotoxicity assay: cell viability test

To assess cell viability, a colorimetric assay based on the MTS-PMS assay (Promega Corp., Madison, WI, USA) was adopted. CHO-K1 cells maintained in DMEM with 10 % FBS were seeded in 96-well plates (10^4 cells/well, 100 µL per well) and maintained for 24 h at 37 °C with 5 % CO_2. Deionized water (5 µL/mL in culture medium) was used as the vehicle control (VC). After that, the cells were incubated with different concentrations of DOTATATE (0.5, 0.9, 1.8, 3.6 and 7.3 ng/mL) and UBI$_{29\text{-}41}$ (0.3, 0.6, 1.1, 2.3 and 4.5 ng/mL) for 24 h. The cell density was determined by adding 20 µL/well of MTS (2 mg/mL) (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and PMS (0.92 mg/mL) (phenazinemethosulfate) in a 20:1 ratio (v/v). After 2 h of incubation, the absorbance values were obtained by the spectrophotometric reading at 490 nm. Each concentration of the compound was tested in quadruplicate in three independent assays. Results were expressed as the percentage of viable cells, with 100 % referring to control cells. The test compound was not considered to be cytotoxic if the cell viability value was at least 90 % of the untreated or negative controls.

Genotoxicity assay: modified MN test

For the genotoxic evaluation, the MN test was conducted according to the cytokinesis block method (Fenech 2007) to obtain binucleated cells. Thus, 300 µL of cell suspension were seeded directly on sterile glass coverslips (15 × 35 mm, Perfecta), placed on 6-well plates (2.5 × 10^3 cell/well) and maintained for 24 h for adhesion at 37 °C with 5 % CO_2. After this time, the coverslips were washed with 3 mL of PBS (pH 7.4) and 2 mL of DMEM medium was added. After 24 h, the cells were treated with peptides (DOTATATE and UBI$_{29\text{-}41}$) diluted in fresh medium without serum at concentrations of 1/10, 1 and 10 times. Three reference mutagens, mitomycin C (MTMC) and colchicine (COLCH), as direct mutagens, and benzo[a] pyrene (BZP), as an indirect mutagen that requires metabolic activation (hepatic S9), were used. Thus, mitomycin C (2.5 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA, CAS 50-07-7), colchicine (1.1 µg/mL) (Sigma-Aldrich, CAS 64-86-8) and benzo[a] pyrene (0.464 mg/mL) (Sigma-Aldrich, CAS 50-32-8) with hepatic (rat) S9 (0.476 mg/mL) (Moltox, Boone, NC, USA, CAS 11097-69-1) were used as positive controls and NaCl (0.9 %) with and without S9 activation, as negative control, maintained for 4 h at 37 °C with 5 % CO_2. After treatment, cultured cells on coverslips were washed twice with PBS and Cytochalasin-B (CytoB) (4 µg/mL) (Sigma-Aldrich, CAS 14930-96-2) was added. After that, the cultured cells on coverslips were washed with PBS, incubated with isotonic solution (0.9 % NaCl) for 15 min, fixed with 4 % paraformaldehyde in PBS for 15 min, washed three times with PBS and left to dry at room temperature for at least 24 h. Fixed cells on coverslips were stained with 50 µL of acridine orange (100 µg/mL) (Sigma-Aldrich, CAS 10127-02-3), mounted on slides and observed with a fluorescence microscope (Nikon 80i, Tokyo, Japan), through a proper filter set (excitation filter of 450–490 nm, emission filter of 515 nm) at 40× magnification. In this procedure, the MN and nuclei appeared bright green and the cytoplasm red, allowing an unequivocal identification of MN in the cells. The samples were coded and randomly counted by two analysts. A minimum of 1000 binucleated cells exhibiting or not MN were counted per sample, by each analyst, also taking into consideration the number of mono- and multi-nucleated cells to determine the cytokinesis-block proliferation index (CBPI), a parameter that indicates whether the treatments induce cell- cycle disturbances. For this calculation the formula used was: $\text{CBPI} = (\text{M1} + 2 \times \text{M2} + 3 \times \text{M3})/\text{N}$, where M1, M2 and M3 represent the number of mono-, bi- and multinucleated cells, respectively, and N is the total number of binucleated cells scored. For each sample, binucleated cells with MN are counted up to 4 MN in order to discriminate from the potential apoptotic events. Results were expressed as the percentage of binucleated cells with MN. The genotoxicity was classified as severe ($\text{MN}_{\text{frequency}}$ Sample $\geq 3 \times \text{MN}_{\text{frequency}}$ C), mild ($\text{MN}_{\text{frequency}}$ Sample $\geq 2 \times$ C) or non-genotoxic ($\text{MN}_{\text{frequency}}$ Sample $\leq 2 \times \text{MN}_{\text{frequency}}$ C).

Statistical analysis

All results were analyzed using the GraphPad Prism program (version 5.0), which was also utilized for the elaboration of figures and tables. Comparisons between the data were performed using the two-way ANOVA and Bonferroni post-analysis with a limit for statistical significance of $p < 0.05$.

Results

Cytotoxicity assay

Figure 1 shows the viability data obtained for CHO-K1 cells treated with different concentrations of DOTATATE (a) and $\text{UBI}_{29\text{-}41}$ (b). Peptides per se did not induce any cytotoxic effect at the tested concentrations. The statistical analyses showed no significant differences ($p > 0.05$) between the treated samples and the respective controls (without treatment and vehicle).

Genotoxicity assay

The modified MN assay using adherent cells and acridine orange staining improved the identification of MN in binucleated cells, permitting accurate analysis and visualization of cellular events (Fig. 2). Other representative events of the cell cycle can also

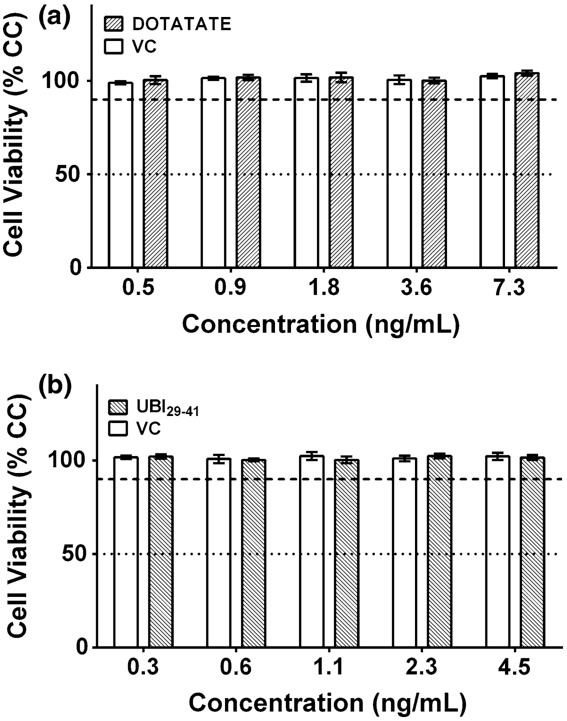

Fig. 1 Cell viability (% of controls) of CHO-K1 cells exposed to different concentrations of DOTATATE (**a**) and $\text{UBI}_{29\text{-}41}$ (**b**). *Dashed* and *dotted lines* indicate 90 and 50 % of control cell viability, respectively. *Columns* represent the means from quadruplicates and bars depict the SEM values. *VC* vehicle control

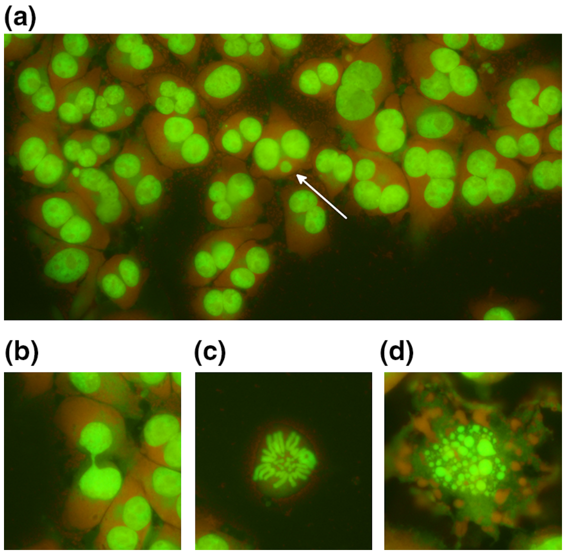

Fig. 2 Microscopic preparations of CHO-K1 cells showing the typical aspect after acridine orange staining. **a** Binucleated cells and the arrow indicate a micronucleus inside of the binucleated cell; **b** nucleoplasmatic bridge; **c** metaphase and **d** cell exhibiting an apoptotic morphology. *Bars* represent 15 µm

be observed, such as nucleoplasmic bridges (NPB) (2b), metaphases (2c) and cells with apoptotic morphology (2d).

The frequency of binucleated cells with MN was adopted as the main parameter for genotoxic evaluation (Fig. 3). Table 1 summarizes the data concerning the percentage of binucleated cells with MN treated with DOTATATE and UBi$_{29\text{-}41}$, with or without metabolic activation, with the corresponding p values.

CHO-K1 cells treated with DOTATATE and UBI$_{29\text{-}41}$ showed no difference from the controls ($p > 0.05$) and, consequently, the peptides can be considered to be non-genotoxic (≤2 × controls) for all tested concentrations, even at higher concentrations corresponding to ten times the maximum doses administered to adults. Also, the negative control (NaCl) did not induce significant changes for the two tested peptides (carrier molecule) when compared to the controls ($p > 0.05$). However, CHO-K1 cells treated with positive controls (MTCC, BZP and COLCH) showed a significant increase in % BNCMN compared to the controls, especially MTCC and BZP, which showed highly a significant

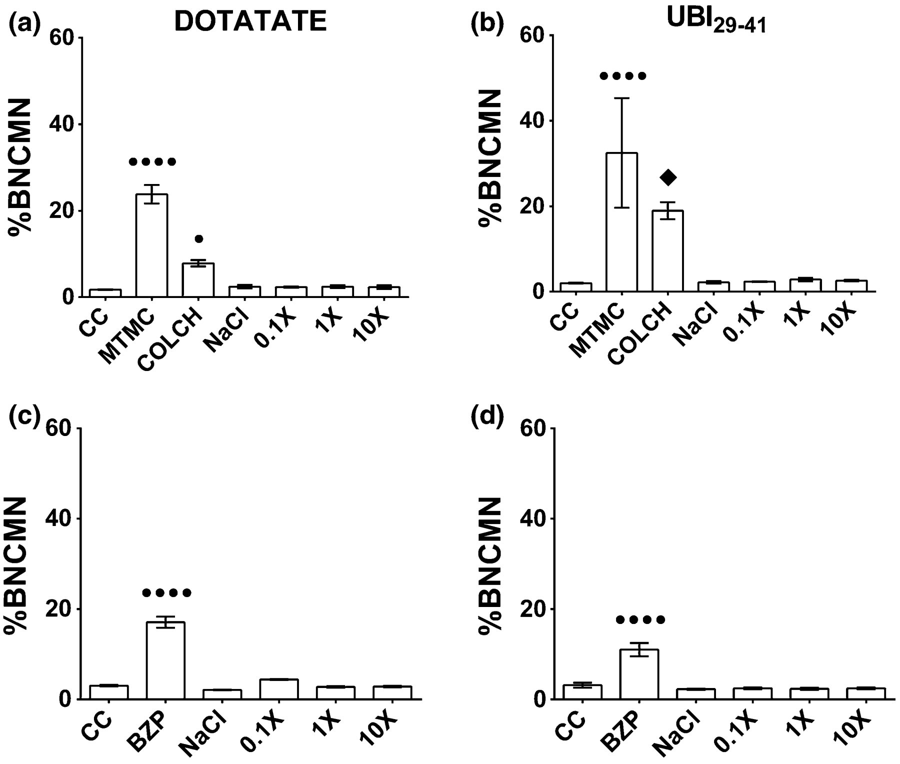

Fig. 3 Percentages of binucleated cells with micronuclei (%BNCMN) in CHO-K1 cells treated with DOTATATE (**a**) or UBI-$_{29\text{-}41}$ (**b**) without S9 and with S9 activation (**c**, **d**). Only positive controls showed significant differences in relation to controls. ●●●● $p < 0.0001$; ●● $p < 0.01$; ♦ $p < 0.05$. *Columns* represent means from duplicates and bars depict SEM values. Concentrations of peptides: ×0.1 = 0.72 ng/mL, ×1 = 7.2 ng/mL, ×10 = 72.0 ng/mL to DOTATATE and ×0.1 = 0.45 ng/mL, ×1 = 4.5 ng/mL, ×10 = 45 ng/mL to UBI$_{29\text{-}41}$

Table 1 Percentages of binucleated cells bearing micronuclei observed in cultures treated with DOTATATE or UBI$_{29\text{-}41}$ and in positive and negative control cultures

	DOTATATE				UBI$_{29\text{-}41}$			
	(−) S9	p value	(+) S9	p value	(−) S9	p value	(+) S9	p value
Control	1.74 ± 0.05	–	3.03 ± 0.15	–	2.02 ± 0.09	–	3.146 ± 0.55	–
NaCl	2.45 ± 0.42	>0.99	2.1 ± 0.02	>0.99	2.2 ± 0.31	=0.98	2.25 ± 0.13	=0.34
MTMC	23.82 ± 2.15	<0.0001	–	–	32.48 ± 12.83	<0.0001	–	–
COLCH	7.84 ± 0.73	=0.0119	–	–	18.96 ± 1.99	=0.0184	–	–
BZP	–	–	17.09 ± 1.21	<0.0001	–	–	11.03 ± 1.47	<0.0001
0.1×	2.31 ± 0.15	>0.99	4.40 ± 0.07	=0.5313	2.34 ± 0.08	=0.96	2.45 ± 0.18	=0.46
1×	2.44 ± 0.35	>0.99	2.75 ± 0.58	>0.99	2.854 ± 0.39	=0.90	2.32 ± 0.22	=0.38
10×	2.31 ± 0.42	>0.99	2.85 ± 0.16	>0.99	2.61 ± 0.16	=0.93	2.43 ± 0.17	=0.45

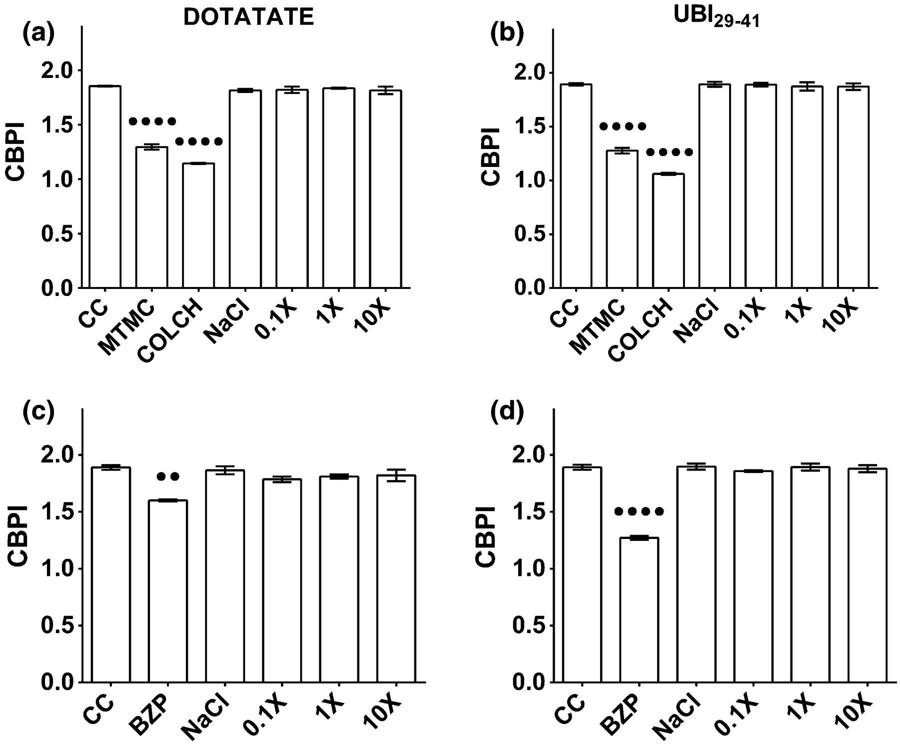

Fig. 4 Cytokinesis-block proliferation index (CBPI) obtained in CHO treated with DOTATATE or UBI-$_{29\text{-}41}$ without (**a**, **b**) or with (**c**, **d**) S9 activation. Only positive controls showed significant differences in relation to controls. ●●●● $p < 0.0001$; ●● $p < 0.01$. *Columns* represent means from duplicates and *bars* depict SEM values. Concentration of peptides: ×0.1 = 0.72 ng/mL, ×1 = 7.2 ng/mL, ×10 = 72.0 ng/mL to DOTATATE and ×0.1 = 0.45 ng/mL, ×1 = 4.5 ng/mL, ×10 = 45 ng/mL to UBI$_{29\text{-}41}$

difference ($p < 0.0001$) in relation to the respective controls.

Cytokinesis-blocking proliferation index: CBPI

Counts of mono-, bi- and multinuclear cells were used in the CBPI calculations. Indexes obtained from cultures exposed to DOTATATE without (a) or with (c) S9, or to UBI-$_{29\text{-}41}$ (b,d), are shown in Fig. 4. Cultures treated with positive controls (MTMC, COLCH and BZP) showed significant decreases of the indexes, and samples treated with DOTATATE or UBI-$_{29\text{-}41}$ showed values with no statistical difference from the negative controls (CC and NaCl 0.9 %).

Comparisons between two independent analysts

After coding and randomization, the microscopic preparations from two independent experiments with UBI$_{29\text{-}41}$ were analyzed by two individual analysts.

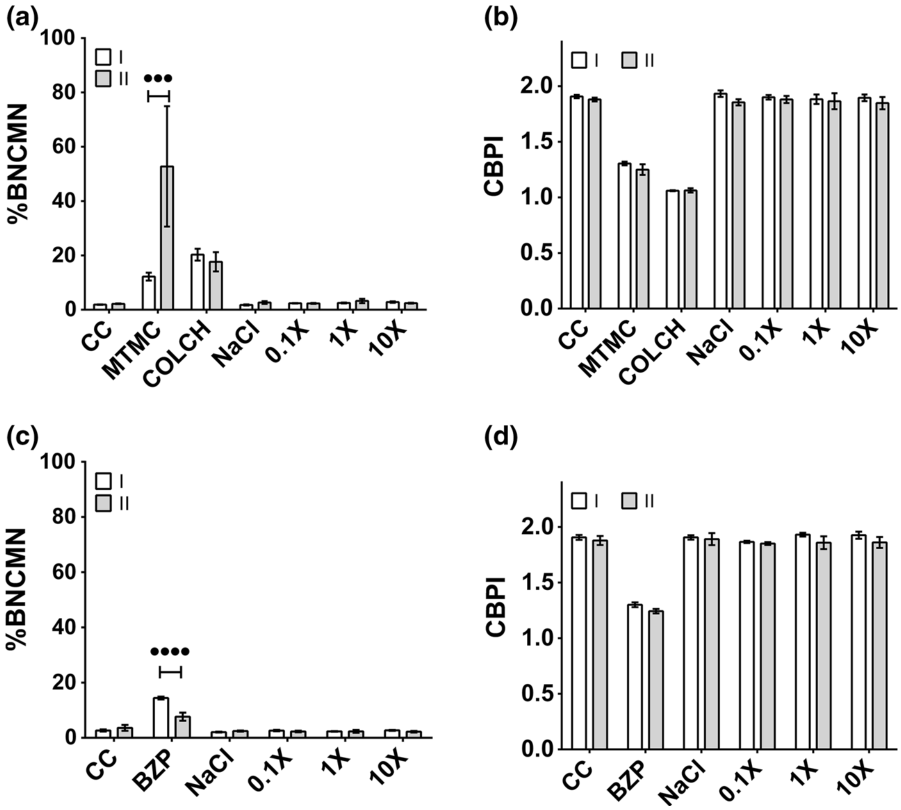

Fig. 5 Statistical differences between scorings from experiments using UBI-$_{29\text{-}41}$ in CHO-K1 cells performed by two independent analysts (I and II): **a**, **b** without S9; **c**, **d** with S9. ●●●● $p < 0.0001$; ●●● $p < 0.001$. Concentration of UBI$_{29\text{-}41}$: ×0.1 = 0.45 ng/mL, ×1 = 4.5 ng/mL, ×10 = 45 ng/mL

Percentages of BNCMN (a,c) and CBPI (b,d) are shown in Fig. 5. Significant differences could be observed in (a) MTMC cultures ($p < 0.001$) and for (c) BZP ($p < 0.0001$). All other tested samples showed no difference between the scorings by analysts I and II, including in the CBPI calculation experiments.

Discussion

Micronuclei scoring is a widely adopted method for determining whether any compound, cytotoxic or not, has the potential to induce double strand breaks (DSB) in the DNA of cells. To assess the genotoxicity of a substance, MN scoring is performed routinely, with great advantage over dicentric-ring chromosome analysis for biological dosimetry of ionizing radiation, although its occurrence is not specific to radiation-induced DSB (De Lemos Pinto et al. 2010). As a toxicology endpoint, MN scoring can be confused by metabolic and/or systemic conditions, such as those due to nutritional causes (Ferguson and Fenech 2012; Nair-Shalliker et al. 2012). Considering some restrictions (Elhajouji et al. 2011), MN scoring from collected samples such as peripheral blood lymphocytes, buccal exfoliated cells (Naga 2016) or urine-derived cells (Nersesyan et al. 2014) can be performed to detect exposures to toxic substances (Demarini 2013) or cancer risk (Bonassi et al. 2011).

Beyond its capabilities to detect genotoxic damage in biological samples, the assay can be applied to the in vitro testing of pharmaceutical compounds. Radiopharmaceuticals and biologically active compounds (such as the peptides used in this study) could be tested following the same protocols. Induction of MN in vitro by biologically active molecules conjugated to PET-emitter isotopes could be detected and related to other endpoints for DNA breakages, such as 53BP1 assembly at break sites (Kashino et al. 2014). This work chose two peptides that are core elements of widely used radiopharmaceuticals, and the lack of cyto/genotoxicity was expected, as the experiments indeed showed.

Because the components of the already marketed products DOTATATE and UBI$_{29\text{-}41}$ were assayed for cytotoxicity only up to the calculated in vitro equivalents of their recommended concentrations in adults, used as the rational dilution basis for genotoxicity experiments. The test system (hamster vary cells) does not express somatostatin receptors, and thus DOTATATE should not be able to bind and/or be internalized by cells, which might explain the lack of in vitro toxicity. In a clinical study, ^{68}Ga-labelled DOTATATE did not induce remarkable toxicity in patients that received up to 50 µg per injection, a higher concentration than those adopted for this study (Deppen et al. 2016). In same way, UBI$_{29\text{-}41}$ should not be internalized by cells due to its lack of bacterial peptidic motifs that would bind to the test substance. Other authors reported cytotoxicity in human hepatic L02 cells only at concentrations above 4 µM (about 670 ng of peptide in 100 µL) (Liu and Gu 2013), a concentration much higher than the used in the present experiments.

For genotoxicity assessment, the maximum concentrations were calculated using the maximum feasible dose concept, as stated in the Materials and Methods section. Previous studies showed that the peptide DOTATATE alone (without radioactive conjugation) did not induce significant genotoxic damage in human lymphocytes in vitro, at concentrations up to 1700 ng/mL (IAEA 2007), higher than the concentrations of this study. No consistent data on the genotoxicity potential of UBI$_{29\text{-}41}$ was found or analyzed by the present study. In this way, one could consider that the chosen concentration range is acceptable for the conclusions of this study.

Acridine orange staining is far from an innovation in MN scoring techniques. A very consistent reference base is available (Çavaş 2008; Heddle et al. 2011; Polard et al. 2011). Nevertheless, its utilization can be preferable in many preparations. The most relevant among the discrete proposals employed in the methodology would be the cell preparation made directly on coverslips. Classical preparation methods can tend to overspread the cells on slides, increasing the scoring time. For toxicology studies, collected cell suspensions (or non-adherent cells) can be more easily analyzed if laid on slides through cytocentrifugation, as in "Cytome Assays" (Fenech 2007; Salimi et al. 2016). Using the same principles, cells can be grown directly on the substrate, preventing dispersion events. These principles are also applicable to innovative approaches for assessing in vitro genotoxicity. Micronuclei scoring of cells grown on microplates (Fenech et al. 2013; Bernardi et al. 2014) also promotes the accumulation of events, which improves visualization and image acquisition. However, these systems are generally not affordable for small test facilities.

Our work proposes modifications of a traditional method that were successfully adopted in GLP routines. As expected, only positive genotoxic controls could induce MN formation in cells. Thus, this work is in consonance with the effects of the peptides already described during safety tests. Inter-analyst evaluation showed good concordance between readings, despite some inconsistencies for MTMC or BZP-treated cultures. It should be possible to reduce this effect by decreasing the time between the separate analyses, but further investigation may be necessary to evaluate this hypothesis. A study with a larger number of samples and analysts would be a reliable way to test if differences could be credited to degradation of fixed material over time, or to real differences in the analyses. However, the CBPI calculations did not show significant differences between counts by the two analysts, perhaps suggesting a relatively acceptable level of preservation of fixed material on coverslips. Apart from these goals, the method also would allow compatibility with image analysis, through manual or automatic acquisition, further expanding its capabilities. Metaphases, and mainly nucleoplasmic bridges, could be observed clearly in this study, and their detection could be profitably used in other studies as related by another study (Tian et al. 2016).

Conclusion

DOTATATE and Ubiquicidin$_{29\text{-}41}$ did not induce any detectable in vitro cytotoxicity or genotoxicity in our test system under concentrations up to ten times the maximum recommended doses per patient. Modifications of the micronucleus scoring technique, including cell culturing directly on slides and optimization of acridine orange staining, could be carried out in a GLP in compliance with regulatory purposes.

Acknowledgments Ivette Zegarra Ocampo was a National Council for Scientific and Technological Development (CNPq) fellow (130778/2014-1). Camila Ayala Lira da Cruz was a PIBIC/PROBIC (CNPq) fellow (161411/2014-2). The authors wish to thank Drs. Elaine Bortoleti de Araújo e Maria Teresa

Coulturato (IPEN—Center of Radiopharmacy—Quality Control Management) for valuable help.

References

Akhtar MS, Qaisar A, Irfanullah J et al (2005) Antimicrobial Peptide 99mTc-Ubiquicidin$_{29–41}$ as human infection-imaging agent: clinical trial. J Nucl Med 46:567–573

Bernardi M, Adami V, Albiero E et al (2014) Absence of micronucleus formation in CHO-K1 cells cultivated in platelet lysate enriched medium. Exp Toxicol Pathol 66:111–116. doi:10.1016/j.etp.2013.11.001

Bonassi S, El-Zein R, Bolognesi C, Fenech M (2011) Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26:93–100. doi:10.1093/mutage/geq075

Casar B, Lopes C, Drljević A et al (2016) Medical physics in Europe following recommendations of the international atomic energy agency. Radiol Oncol 50:64–72. doi:10.1515/raon-2016-0004

Çavaş T (2008) In vivo genotoxicity of mercury chloride and lead acetate: micronucleus test on acridine orange stained fish cells. Food Chem Toxicol 46:352–358. doi:10.1016/j.fct.2007.08.015

Çelik A, Öğenler O, Çömelekoğlu Ü (2005) The evaluation of micronucleus frequency by acridine orange fluorescent staining in peripheral blood of rats treated with lead acetate. Mutagenesis 20:411–415. doi:10.1093/mutage/gei055

Cescato R, Schulz S, Waser B et al (2006) Internalization of sst2, sst3, and sst5 receptors: effects of somatostatin agonists and antagonists. J Nucl Med 47:502–511

Chaturvedi S, Mishra AK (2016) Small Molecule Radiopharmaceuticals—a review of current approaches. Front Med 3:1–18. doi:10.3389/fmed.2016.00005

De Lemos Pinto MMP, Santos NFG, Amaral A (2010) Current status of biodosimetry based on standard cytogenetic methods. Radiat Environ Biophys 49:567–581. doi:10.1007/s00411-010-0311-3

Demarini DM (2013) Genotoxicity biomarkers associated with exposure to traffic and near-road atmospheres: a review. Mutagenesis 28:485–505. doi:10.1093/mutage/get042

Deppen SA, Liu E, Blume JD et al (2016) Safety and efficacy of 68Ga-DOTATATE PET/CT for diagnosis, staging and treatment management of neuroendocrine tumors. J Nucl Med 57:708–715. doi:10.2967/jnumed.115.163865

Elhajouji A, Lukamowicz M, Cammerer Z, Kirsch-Volders M (2011) Potential thresholds for genotoxic effects by micronucleus scoring. Mutagenesis 26:199–204. doi:10.1093/mutage/geq089

Fenech M (2000) The in vitro micronucleus technique. Mutat Res Fundam Mol Mech Mutagen 455:81–95. doi:10.1016/S0027-5107(00)00065-8

Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc 2:1084–1104. doi:10.1038/nprot.2007.77

Fenech M, Kirsch-Volders M, Rossnerova A et al (2013) HUMN project initiative and review of validation, quality control and prospects for further development of automated micronucleus assays using image cytometry systems. Int J Hyg Environ Health 216:541–552. doi:10.1016/j.ijheh.2013.01.008

Ferguson LR, Fenech MF (2012) Vitamin and minerals that influence genome integrity, and exposure/intake levels associated with DNA damage prevention. Mutat Res Fundam Mol Mech Mutagen 733:1–3. doi:10.1016/j.mrfmmm.2012.03.009

Harapanhalli RS (2010) Food and drug administration requirements for testing and approval of new radiopharmaceuticals. Semin Nucl Med 40:364–384. doi:10.1053/j.semnuclmed.2010.05.002

Heddle JA, Fenech M, Hayashi M, MacGregor JT (2011) Reflections on the development of micronucleus assays. Mutagenesis 26:3–10. doi:10.1093/mutage/geq085

IAEA (2007) Comparative evaluation of therapeutic radiopharmaceuticals. IAEA, Vienna

Johnbeck CB, Knigge U, Kjær A (2014) PET tracers for somatostatin receptor imaging of neuroendocrine tumors: current status and review of the literature. Future Oncol 10:2259–2277. doi:10.2217/fon.14.139

Kashino G, Hayashi K, Douhara K et al (2014) Comparison of the biological effects of (18)F at different intracellular levels. Biochem Biophys Res Commun 454:7–11. doi:10.1016/j.bbrc.2014.09.136

Liu C, Gu Y (2013) Noninvasive optical imaging of Staphylococcus aureus infection in vivo using an antimicrobial peptide fragment based near-infrared fluorescent probes. J Innov Opt Health Sci 06:1350026. doi:10.1142/S1793545813500260

Lupetti A, de Boer MG, Erba P et al (2011) Radiotracers for fungal infection imaging. Med Mycol 49:S62–S69. doi:10.3109/13693786.2010.508188

Naga MBSS (2016) Buccal micronucleus cytome assay in sickle cell disease. J Clin Diagn Res 10:ZC62–4. doi:10.7860/JCDR/2016/19984.7998

Nair-Shalliker V, Armstrong BK, Fenech M (2012) Does vitamin D protect against DNA damage? Mutat Res Fundam Mol Mech Mutagen 733:50–57. doi:10.1016/j.mrfmmm.2012.02.005

Nersesyan A, Kundi M, Fenech M et al (2014) Micronucleus assay with urine derived cells (UDC): a review of its application in human studies investigating genotoxin exposure and bladder cancer risk. Mutat Res Rev Mutat Res 762:37–51. doi:10.1016/j.mrrev.2014.04.004

Nilica B, Waitz D, Stevanovic V et al (2016) Direct comparison of 68Ga-DOTA-TOC and 18F-FDG PET/CT in the follow-up of patients with neuroendocrine tumour treated with the first full peptide receptor radionuclide therapy cycle. Eur J Nucl Med Mol Imaging 43(9):1585–1592. doi:10.1007/s00259-016-3328-2

OECD (2010) Oecd guideline for the testing of chemicals: invitro mammalian cell micronucleus test. OECD, Paris

Okarvi SM (2008) Peptide-based radiopharmaceuticals and cytotoxic conjugates: potential tools against cancer. Cancer Treat Rev 34:13–26. doi:10.1016/j.ctrv.2007.07.017

Ostovar A, Assadi M, Vahdat K et al (2013) A pooled analysis of diagnostic value of (99 m)Tc-ubiquicidin (UBI) scintigraphy in detection of an infectious process. Clin Nucl Med 38:413–416. doi:10.1097/RLU.0b013e3182867d56

Polard T, Jean S, Merlina G et al (2011) Giemsa versus acridine orange staining in the fish micronucleus assay and validation for use in water quality monitoring. Ecotoxicol Environ Saf 74:144–149. doi:10.1016/j.ecoenv.2010.08.005

Salimi M, Broumand B, Mozdarani H (2016) Association of elevated frequency of micronuclei in peripheral blood lymphocytes of type 2 diabetes patients with nephropathy complications. Mutagenesis. doi:10.1093/mutage/gew029

Santos GS, Tsutsumi S, Vieira DP et al (2014) Effect of Brazilian propolis (AF-08) on genotoxicity, cytotoxicity and clonogenic death of Chinese hamster ovary (CHO-K1) cells irradiated with 60Co gamma-radiation. Mutat Res Genet Toxicol Environ Mutagen 762:17–23. doi:10.1016/j.mrgentox.2013.11.004

Sobol Z, Homiski ML, Dickinson DA et al (2012) Development and validation of an in vitro micronucleus assay platform in TK6 cells. Mutat Res Genet Toxicol Environ Mutagen 746:29–34. doi:10.1016/j.mrgentox.2012.02.005

Speit G, Zeller J, Neuss S (2011) The in vivo or ex vivo origin of micronuclei measured in human biomonitoring studies. Mutagenesis 26:107–110. doi:10.1093/mutage/geq061

Tian X-L, Zhao H, Cai T-J et al (2016) Dose-effect relationships of nucleoplasmic bridges and complex nuclear anomalies in human peripheral lymphocytes exposed to 60Co γ-rays at a relatively low dose. Mutagenesis 31:425–431. doi:10.1093/mutage/gew001

van Es SC, Venema CM, Glaudemans AWJM et al (2016) Translation of new molecular imaging approaches to the clinical setting: bridging the gap to implementation. J Nucl Med 57:96S–104S. doi:10.2967/jnumed.115.157974

Westerink WMA, Schirris TJJ, Horbach GJ, Schoonen WGEJ (2011) Development and validation of a high-content screening in vitro micronucleus assay in CHO-k1 and HepG2 cells. Mutat Res Genet Toxicol Environ Mutagen 724:7–21. doi:10.1016/j.mrgentox.2011.05.007