https://doi.org/10.31925/farmacia.2021.5.8 **ORIGINAL ARTICLE**

PRECLINICAL EVALUATION OF THE CYTOTOXIC EFFECTS INDUCED BY ^{68}Ga-NODAGA-c(RGDfK) AND ^{68}Ga-DOTA-c(RGDfK) IN MURINE MALIGNANT MELANOMA

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Abstract

Targeting alpha(v)beta(3) integrin receptors cells using ^{68}Ga-labelled RGD peptides could be a promising approach for Positron Emission Tomography (PET). Data shows that this medical investigation causes minimal damage to adjacent tissues. However, little information is available concerning the effects that may occur in tumour tissue after ^{68}Ga exposure. Therefore, in this paper we investigated some of the biological effects induced by ^{68}Ga-NODAGA-c(RGDfK) and ^{68}Ga-DOTA-c(RGDfK) in melanoma bearing mice, evaluating different parameters underlying melanoma progression such as: (1) apoptotic activity, (2) cell cycle phases and (3) oxidative stress levels evaluated by lipid peroxidation and total thiols concentrations in cell lysates. The two radiolabelled compounds induced increases of apoptotic events as well as G2/M cell cycle blockage, in tumour cells. Conversely, the two radiolabelled compounds induced no significant changes in the oxidative stress. The results demonstrated the ability of ^{68}Ga-labelled compounds to exert inhibitory effects on tumour cells, manifested both by apoptosis induction and cell cycle blockage. We consider that the results will help to a better understanding of radiation biology revealing some of the cellular changes associated with medical imaging procedures. These results are also promising for further *in vitro* and *in vivo* evaluation of Peptide Receptor Radionuclide Therapy.

Rezumat

Țintirea receptorilor celulari ai integrinei alfa(v)beta(3) utilizând peptide RGD marcate cu ^{68}Ga ar putea reprezenta un instrument promițător pentru tomografia cu emisie de pozitroni (PET). Datele arată că o astfel de investigație medicală produce leziuni minime țesuturilor adiacente. Cu toate acestea, există puține informații la ora actuală despre efectele care pot apărea în țesutul tumoral după expunerea la ^{68}Ga. Prin urmare, în această lucrare am investigat unele dintre efectele biologice induse de ^{68}Ga-NODAGA-c(RGDfK) și ^{68}Ga-DOTA-c(RGDfK) la șoareci purtători de melanom, evaluând diferiți parametri care stau la baza progresiei tumorale a melanomului, precum: (1) activitatea apoptotică, (2) fazele ciclului celular si (3) nivelurile de stres oxidativ - evaluate prin peroxidarea lipidelor și prin concentrațiile totale de tioli, din lizatele celulare. S-a observat că cei doi compuși radiomarcați au indus creșteri ale evenimentelor apoptotice precum și blocarea ciclului celular în faza G2/M. În schimb, aceștia nu au indus modificari semnificative la nivelul statusului stresului oxidativ. Rezultatele au demonstrat astfel capacitatea compușilor marcați cu ^{68}Ga de a exercita efecte inhibitorii asupra celulelor tumorale, bazate atât pe inducerea apoptozei cât și prin blocarea ciclului celular. Consideram că rezultatele vor ajuta la o mai bună înțelegere a biologiei radiațiilor, relevând unele modificări celulare asociate cu procedurile de imagistică medicală. Rezultatele sunt, de asemenea, promițătoare pentru evaluarea ulterioară *in vitro* și *in vivo* a terapiei bazate pe radionuclizi a receptorilor peptidici.

Keywords: B16/F10 melanoma, ^{68}Ga-NODAGA-c(RGDfK), ^{68}Ga-DOTA-c(RGDfK), alpha (v)beta(3) receptors, PET

Introduction

The malignant melanoma is one of the most aggressive forms of cancer with a low survival rate, worldwide registering more than 280,000 new cases and 60,000 deaths *per* year [1]. Due to its resistance to chemotherapy, immunotherapy and external beam radiotherapy, it is necessary to find new therapeutic agents (conventional chemotherapeutics or molecularly targeted drugs) and strategies for the melanoma treatment. Moreover, early detection of this cancer may be crucial for the success of patient treatment [4].

Various targets, such as cell receptors known to be overexpressed during the tumour progression have been widely studied for use in melanoma detection and therapy [16]. For instance, alpha(v)beta(3) integrin receptors were found to be lowly expressed on the mature epithelial cells and resting endothelial cells, but upregulated on activated endothelial cells during melanoma neovascularization [10, 14]. Studies utilizing alpha(v)beta(3) integrin inhibitory peptides, antibodies or small molecular compounds indicate that alpha(v)-beta(3) integrin indeed is required for tumour

endothelial cell survival; therefore its inhibition may lead to the regression of tumour vasculature [6]. Several radiolabelled alpha(v)beta(3) inhibitors are currently evaluated for tumour imaging and targeted radiotherapy of a wide range of tumours, including melanoma [5]. One of the radiolabelled peptides binding preferentially to alpha(v)-beta(3) receptors and exhibiting very satisfactory pharmacokinetics was shown to be ^{68}Ga-DOTA-RGD [2, 3, 5]. Recently, ^{68}Ga-NODAGA-RGD has also been designed for non-invasive visualization of alpha(v)beta(3) expression, showing improved imaging properties compared to ^{68}Ga-DOTA-RGD [17].

The applications of ^{68}Ga-labelled peptides have been developing lately and may represent a very promising class of radiopharmaceuticals for PET that combine several advantages, including: wide availability of ^{68}Ga (from in-house generator); rapid pharmacokinetics and good tumour targeting characteristics; without notable side effects, allowing several administrations in patients *per* day [8, 17]. Also, it was showed that the radio-isotope ^{68}Ga used in PET investigations causes minimal damage to adjacent tissues and can be easily eliminated from the body. The studies showed that the level of DNA damage observed in the blood of patients examined by PET is close to the back-ground level found in the blood of a healthy volunteer suggesting no adverse effects on the patients [12]. However, currently there is little information on the effects that occur in tumour tissue after ^{68}Ga exposure, in PET investigations.

In our previous study we focused on the biological effects induced *in vitro* by different ^{68}Ga-radio-labelled peptides developed for PET investigation on several human and murine tumour cell lines [9].

In this paper, we expanded our research *in vivo* exploring the biological effects of two selected ^{68}Ga-peptide conjugates employing distinct chelators (DOTA-RGD *versus* NODAGA-RGD) after their intravenous administration in B16/F10 melanoma-bearing mice. We investigated parameters under-lying melanoma progression: the apoptotic events, cell cycle phases and oxidative stress levels.

Materials and Methods

Chemicals and radio-chemicals: NODAGA-c(RGDfK) (Cyclo [-Arg-Gty-Asp-dPhe-Lys(NODAGA)-]) and DOTA-c(RGDfK) (Cyclo [-Arg-Gly-Asp-dPhe-Lys (DOTA)-]) were purchased from PiCHEM (Graz, Austria). The peptide compounds were radiolabelled with ^{68}Ga fraction of the highest activity, eluted from a ^{68}Ge/^{68}Ga generator system (925 MBq, ITG, Germany) in IFIN-HH, to obtain ^{68}Ga-NODAGA-c(RGDfK) and ^{68}Ga-DOTA-c(RGDfK). After synthesis, the radio-labelled peptides were further purified by HPLC, using the eluate in PBS of high radiochemical purity (more than 98%) for *in vivo* studies.

Animal model: B16 melanoma cells (2 x 10^6 cells/ 200 µL PBS) were s.c. injected in the dorsal region of 6 - 8-week-old males C57BL/6 mice. Then, animal health was checked at least twice weekly by visual inspection of general appearance, behaviour, tumour inoculation site and by weighing. Mice with tumour volume about 50 ± 5 mm^3 received 10 MBq (10 µg) intravenous injections of ^{68}Ga-NODAGA-c(RGDfK) (group 1), or ^{68}Ga-DOTA-c(RGDfK) (group 2) *via* the tail vein. The control group did not receive any of the above treatments. Subsequently, the all three groups of mice were euthanized and the tumour tissues were aseptically collected. All experiments have been carried out in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposed.

Preparing a single cell suspension from tissue tumour: After sampling the tumour tissues are rinsed to clean any blood or other unwanted material, then chopped and dispersed with scissors in phosphate buffer saline (PBS) until complete disintegration. The obtained cell suspension is filtered through special sieves (50 µm mesh) to cell agglutinates removing. Then, cold PBS is added over the pellet, adjusting the cell concentration to 1 x 10^6 cells/ml. After successive washes with PBS and centrifugation at 300g, 2 mL of 70% cold ethanol is added drop by drop under continuous stirring, on the pellet. At least 24 h after ethanol removal, the cell suspensions are treated with different reagents in order to evaluate the cell cycle phases, apoptosis status and oxidative stress to further quantify the effects of the two injected radiochemicals on tumour cells.

Apoptosis Assays: According to the Abcam manufacturer's protocol, 100 µL suspension (1 x 10^5 cells) was stained with 5 µL fluorescein isothiocyanate (FITC) Annexin V and 5 µL propidium iodide (PI) and incubated in the dark at room temperature (RT) for 5 min. The samples were analysed on FACSCalibur Flow Cytometer (Becton Dickinson, USA) by Cellquest software. The number of apoptotic and/or necrotic cells was calculated using WinMDI 2.9 software. There were identified and quantified by Annexin-V/PI double staining assay the early apoptotic cells (lower right quadrant), living cells (lower left quadrant), late apoptotic cells (upper right quadrant) and necrotic cells (upper left quadrant).

Cell Cycle Assays: Subsequenly, 50 µL suspensions (1 x 10^5 cells) were treated with 400 µl RNAse A (Sigma; 1.25 mg/mL stock) after overnight fixation in 70% ethanol. Thereafter, were stained with 100 µL propidium iodide at RT, in the dark. The nuclear DNA content was analysed on a FACSCalibur Flow Cytometer by CellQuest software. Data were analysed on a histogram in ModFIT LT software, depending on the fluorescence emission pulse (FL2-Area) and cell number, only for non-aggregated cells. There were

established the percentage of cells at G0/G1, S and G2/M phase using propidium iodide staining.

Biochemical Assays: The cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% TritonX-100, and 1% sodium deoxycholate with sonication (20 kHz, 10 s, twice) in an ultrasonic homogenizer (UH-50, Tokyo, Japan). Lipid peroxidation products were measured using thiobarbituric acid reactive substances (TBARS reaction) and total thiols were monitored by Schosinsky assay [13]. Spectrophotometric assays were performed with the AnalyticYena spectrophotometer and the winASPECT software (Yena, Germany).

Results and Discussion

In the recent years, PET has been increasingly used in cancer investigation, both for diagnosis and staging, while also providing prognostic information. It is known that the radioisotopes used in PET investigations cause minimal damage to adjacent tissues and can be easily eliminated from the body [12]. However, there is little information about the biological effects occurring directly in tumour tissue after exposure to these radionuclides. Studies on a wide range of cell lines derived from human tumours have shown that different radiation agents can be able to arrest the cell cycle at a specific checkpoint and thereby to induce cell death by apoptosis. Though the apoptotic activity and cell cycle phases are considered important in cancer progression, these features have not been much studied in melanoma [7, 15].The present study investigated *in vivo* the biological effects induced by ^{68}Ga-NODAGA-c(RGDfK) and ^{68}Ga-DOTA-c(RGDfK), intravenously administered in C57BL/6 mice bearing B16/F10 melanoma. Different parameters underlying melanoma progression such as: the apoptotic activity and cell cycle phases (Go/G1, S, G2+M), as well as the oxidative stress levels – lipid peroxidation and total thiols concentrations in cell lysates were used to determine the radiobiological responses of cancer cells. We investigated in what extent the effects were due to the induction of apoptosis or cell cycle blockage in relation with oxidative stress parameters.

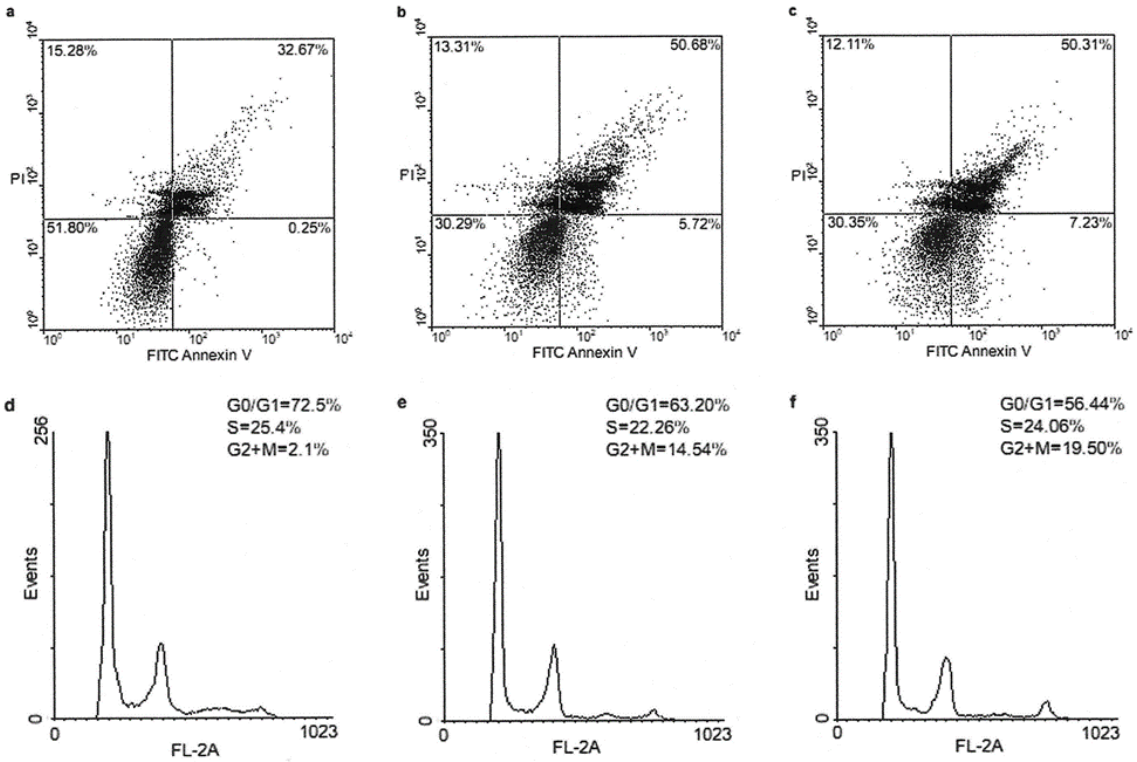

Figure 1.

The effect of ^{68}Ga-DOTA-c(RGDfK) and ^{68}Ga-NODAGA-c(RGDfK) on apoptosis (b and c) and cell cycle distribution (e and f) in mice bearing B16/F10 melanoma. Untreated mice bearing B16/F10 melanoma were used as control (a and d)

Apoptotic activity level in the B16/F10 melanoma cells

The two radiolabelled compounds, ^{68}Ga-DOTA-c(RGDfK) and ^{68}Ga-NODAGA-c(RGDfK) promoted apoptosis of B16/F10 tumour cells. The apoptotic events in tumours increased with 5.72 and 7.23% (Figures 1b and 1c) compared with control (Figure 1a). The percentage of living cells decreased to 30.29% after ^{68}Ga-DOTA-c(RGDfK) treatment and to 30.35% after ^{68}Ga-NODAGA-c(RGDfK) treatment,

compared with the control group. Instead, necrosis registered no important differences between the treated and untreated tumours, the percentage of necrotic cells being 15.28, 13.31 and 12.11%, respectively. These data indicated that the two radiolabelled compounds induced death of tumour cells by apoptosis.

The cell cycle alteration level in the B16/F10 melanoma cells

As shown in the Figures 1e and 1f, the G2/M phase of tumour cells was slightly affected by the two radio-labelled compounds when compared to the control group (Figure 1d). We observed cell cycle blockage in G2/M . G2/M-phase increased to 14.54 and 19.50% (Figures 1e and 1f) compared with control (Figure 1d). However, G2/M accumulation was not been accompanied by a corresponding reduction of S phase. As shown in Figure 1e and 1f, the percentage of B16/F10 cells in G1 phase after the treatment was lower, compared to the percentage in G1 for the control group. These results demonstrated that the two radiolabelled compounds could arrest the cell cycle in the G2/M phase.

In the previous experimental study performed this time *in vitro* on B16/F10 melanoma cell line [9], we investigated whether alpha(v)beta(3) integrin receptor binding ^{68}Ga-labelled RGD peptides synthesized for PET diagnostic applications could influence growth of tumour cells as a result of apoptosis promoting and/or cell cycle dysregulation. Briefly, we investigated in what extent the effects of ^{68}Ga-labelled peptides were due to induction of apoptosis or cell cycle arrest in tumour cells in connection with oxidative stress generation. The results showed a slight increase in apoptosis induced by the ^{68}Ga-NODAGA-c (RGDfK) and ^{68}Ga-DOTA-c (RGDfK), comparing with untreated cells (control). Conversely, there were found substantial changes of DNA profile in case of ^{68}Ga-NODAGA-c(RGDfK) with 30.55% of cells blocked in G2/M compared to only 14.70% of cells treated with ^{68}Ga-DOTA-c(RGDfK) and even 1.19% of control. Oppositely, the G0/G1 fraction decreased from 61.11 (control) to 33.42% for the first compound and 42.72% for the second one, without modifying of S-fraction. Starting from the fact that preclinical animal tests have the advantage of allowing melanoma cells to directly establish interactions with the murine stroma including lymphatic and blood vessels, in this paper we investigated *in vivo* growth behaviour and melanoma cell response to radionuclide action. We observed that alpha(v)beta(3) receptor binding ^{68}Ga labelled peptides synthesized for PET diagnostic application, in some extent, affects the *in vivo* growth of B16/F10 melanoma mouse, as a result of apoptosis promotion, especially after administration of ^{68}Ga-NODAGA-c(RGDfK). But this apoptosis promotion is not followed by a significant cell cycle dys-regulation, only 14.54%, respectively 19.50% being blocked.

The oxidative stress level in the B16/F10 melanoma cells

We investigated, *in vivo*, in mice bearing B16/F10 melanoma, before and after ^{68}Ga-labelled peptides administrations: the peroxidation of lipid products and the depletion of the total thiols. It is known that free radicals play an important role in the initiation and promotion of cancer, by affecting the balance between apoptosis and cellular proliferation [7]. An increase in free radicals' level, through an increase in production or a delay, causes DNA, protein or lipid damage that can play a critical role in carcino-genesis, but also, a delicate change in redox conditions can play a role in tumour progression by affecting cell growth or survival [11].

Table I reveals a very slight decrease in the total SH group concentration compared with control group. In addition, the MDA concentration was not significantly decreased following the two radiolabelled compounds treatment. The results showed no significant changes in the increasing of oxidative stress between the two radiolabelled peptides, suggesting their non-oxidative mediated cytotoxicity. In our previous study on the cell lysates, slight increases of reactive oxygen species and, implicitly, of lipid peroxidation and total thiols content were observed following B16/F10 cell line irradiation. Insignificant changes in lipid peroxidation and total thiols content between the two radiolabelled peptides were observed, suggesting their non-oxidative mediated cytotoxicity. In the present study an insignificant increase in total thiol in tissues might be due to increased free radical production, but in amounts that are scavenged by antioxidants, such that the damage is limited to few protein molecules. Our results are consistent with those obtained in other experimental studies, performed *in vitro*, on B16/F10 melanoma cell line [9].

Table I

Oxidative stress assessment *in vivo* in mice bearing B16/F10 melanoma: lipid peroxidation products (TBARS) and total thiols concentrations after the treatment with radiolabelled compounds *vs*. control (untreated cells) are shown

B16/F10	Radiolabelled compound	MDA conc. (µmol/100 mL)	Total SH group conc. (µmol/L)
tumour	control	9.50	246
	^{68}Ga-DOTA-c(RGDfK)	10.11	221
	^{68}Ga-NODAGA-c(RGDfK)	9.83	208

Conclusions

Similar to previous *in vitro* studies, our results showed an increase of apoptosis induced by ^{68}Ga-NODAGA-c(RGDfK) and ^{68}Ga-DOTA-c(RGDfK) comparing with control group. It was also found that the G2/M phase of tumour cells was slightly affected by the two radiolabelled compounds. In contrast, the two radiolabelled compounds did not induce significant changes in oxidative stress. Thus, both ^{68}Ga-NODAGA-c(RGDfK) and ^{68}Ga-DOTA-c(RGDfK) exert inhibitory effects on C57BL/6 mice bearing B16/F10 melanoma, manifested by cell cycle dysregulation and apoptosis induction. The results of this study will help to better understanding of radiation biology, revealing some of cellular changes. Our data suggest the utility of this model for the study of tumour biology and for the validation of tumour imaging modality targeting integrin receptors. Therefore, the two radiolabelled compounds could be used as pre-therapeutic agents in PET to predict the response to peptide receptor radionuclide therapy (PRRT).

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Conflict of interest

The authors declare no conflict of interest.

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