*2019 International Nuclear Atlantic Conference - INAC 2019*
*Santos,SP, Brazil, October 21-25, 2019*
*ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN*
***ISBN:* 978-85-99141-08-3**

# SYNTHESIS OF A 2-NITROIMIDAZOLE-GLYCOPEPTIDE RADIOLABELED WITH $^{68}$GA FOR POSITRON EMISSION TOMOGRAPHY (PET) IMAGING OF TUMOR HYPOXIA

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## ABSTRACT

Hypoxia is a pathological condition characterized by a reduction of oxygen supply to a specific tissue or cell. About 60% of solid tumors in an advanced stage present areas of hypoxia. Tumor-associated hypoxia has been correlated to: 1) tumor aggressiveness; 2) resistance to chemotherapy and radiotherapy; 3) poor prognosis. Thus, the use of non-invasive methods dedicated to assess tumor hypoxic areas are of extremely importance for the treatment of several types of cancers, allowing the use of individualized therapeutic strategies. Here, we developed a new $^{68}$Ga-labeled radiopharmaceutical for positron emission tomography (PET) imaging of tumor hypoxia. The $^{68}$Ga-labelled 2-nitroimidazole derivative was successfully obtained by linking the 2-nitroimidazole acetic acid derivative with a glycopeptide obtained by solid phase synthesis and further conjugated to DOTA-NHS and its identity was confirmed by mass spectrometry. The radiolabeling procedure of $^{68}$Ga-Glycopeptide was optimized regarding the amount of glycopeptide, temperature and time, and was obtained with a high radiochemical purity (96.6± 0.4%). Compared to the standard hypoxic radiopharmaceutical $^{18}$F-FAZA, $^{68}$Ga-Glycopeptide was obtained in a faster way and high radiochemical purity was achieved after radiolabeling procedures. Our new $^{68}$Ga-Glycopeptide may be promising candidate for further evaluation as a potential hypoxia imaging agent. Moreover, the use of $^{68}$Ga as an alternative to $^{18}$F in the development of new tracers for PET imaging is still an advantage because of the use of radionuclide generators instead of costly cyclotron equipment. Additionally, the use of a glycopeptide may allow the development of a kit-type setup that will ease the preparation of the $^{68}$Ga-based agent.

## 1. INTRODUCTION

Cancer is a chronic degenerative process that culminates in the loss of mechanisms that regulate cell cycle and death. In addition, it is considered a public health problem worldwide and its incidence has grown by 20% in the last decade. In Brazil, it is the second cause of death due to illness and the National Cancer Institute estimate is approximately 600 thousand

new cases in 2019 [1]. According to the World Health Organization (WHO) estimates that by the year 2030 there will be 27 million new cases of cancer, 17 million deaths from the disease and 75 million people living with cancer. Developing countries will be the most affected, including Brazil. Currently, cancer is one of the world's major health policy concerns.

Currently, it is known that up to 60% of solid tumors in advanced stages contain regions with low oxygen concentration, also known as hypoxia [2]. Hypoxia causes genomic changes by upregulation of the transcription factor hypoxia inducible factor 1 (HIF-1) and can promote tumor invasiveness and metastasis [3]. Additionally, it leads to decreased sensitivity of tumors to radiation therapy and chemotherapy. Hypoxia results in inefficient transformation of therapeutic radiation induced damage to cytotoxic DNA-strand breaks [4]. The effectiveness of chemotherapy is diminished by limited drug diffusion to hypoxic tumor regions [5] and through activation of multiple molecular mechanisms that decrease the sensitivity of (hypoxic) tumor cells to anti-cancer drugs [6]. Because of these potential effects of hypoxia on tumor biology and therapy, monitoring tumor hypoxia status could provide a valuable tool for treatment planning. Several different methods are in use for the detection and measurement of tumor hypoxia that can be broadly categorized as either invasive techniques, such as the measurement of oxygen partial pressure (pO2) with polarographic electrodes and immunohistochemical (IHC) staining of endogenous and/or exogenous markers of hypoxia, or noninvasive techniques, such as positron emission tomography (PET) imaging using radiolabelled hypoxia markers [7-9].

In clinical studies of tumor hypoxia imaging with PET, probes such as [$^{18}$F]fluoromisonidazole ([$^{18}$F]FMISO), [$^{18}$F]fluoroazomycin arabinoside ([$^{18}$F]FAZA), [$^{18}$F] flortanidazole([$^{18}$F]HX4),[$^{18}$F]2-(2-Nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide ([$^{18}$F]EF5), and [$^{18}$F]-fluoroetanidazole ([$^{18}$F]FETA) have been used [10]. Several hypoxia probes contain 2-nitroimidazole as a functional moiety in their structure [9]. In overview of the uptake and retention mechanisms of hypoxia probes containing 2-nitroimidazole, each probe is passively diffused through the cell membrane owing to the lipophilicity, and once within the intracellular environment, these are reduced into R-NO2 radicals. This process is still reversible, and when the intracellular environment is sufficiently oxygenated, the tracer is not retained and can freely flow back into the extracellular environment. The consequence is the progressive production of R-NHOH compounds that bind covalently to macromolecules and via glutathione conjugation following nitro-group reduction and ultimately retain the tracer within the hypoxic cells [11,12]. Among these hypoxia probes, [$^{18}$F]FMISO remains the most extensively studied; moreover, its feasibility for hypoxia-specific imaging has been extensively shown in both preclinical and clinical settings with different cancer types [13,14]. In spite of its good clinical output, [$^{18}$F]FMISO has been criticized because of its slow accumulation in hypoxic tumors and low tumor-to-background ratios due to non-specific binding resulting from its relatively high lipophilicity [9,15]. To overcome the limitations of [$^{18}$F]FMISO, some next-generation hypoxia PET probes with additional hydrophilic properties, such as [$^{18}$F]FAZA and [$^{18}$F]HX4, were developed to obtain better contrast images by accelerating renal clearance. The biodistribution of [$^{18}$F]FAZA was improved by the addition of a sugar moiety, making it less lipophilic than [$^{18}$F]FMISO[16]. Moreover, [$^{18}$F]FAZA has shown promising results in preclinical and clinical studies based on its selective accumulation in hypoxia tumor cells [17,18]. However, the reproducibility of the PET data for [$^{18}$F]FAZA has not been resolved in hypoxia image-guided radiotherapy [19]. To date, no hypoxia PET probes have been widely adopted for routine clinical practice due to these limitations.

Other radionuclides have also been used, in particular, 68-Gallium due to its availability from a $^{68}Ge/^{68}Ga$ generator. Several research groups are pursuing the development of [$^{68}$Ga]-complexes for hypoxic tumor imaging [20-22]. Mukai et al. [20] reported a DOTA-derivative with two 5-nitromidazol-1-yl moieties coupled through an amide bond. Additionally, Hoigebazar et al. [21,22] developed compounds with a 2-nitroimidazol-1-yl moiety coupled to DOTA or NOTA. Some of these reported [$^{68}$Ga]-complexes showed moderate tumor uptake, high hydrophilicity and a fast clearance from soft tissues. Although authors claim that results obtained are comparable with those for [$^{18}$F]FMISO and [$^{18}$F]FAZA, this comparison was not made under the same experimental conditions and biological models [21]. As a consequence, it is relevant to continue developing new tracers with improved biological properties.

Consequently, we present herein the synthesis of a novel glycopeptide 2-nitroimidazole derivative, containing DOTA-NHS as chelating agent for $^{68}$Ga and the preparation of its corresponding $^{68}$Ga-complexe, [$^{68}$Ga]Glypeptide-2-Nitroimidazole.

## 2. MATERIALS AND METHODS

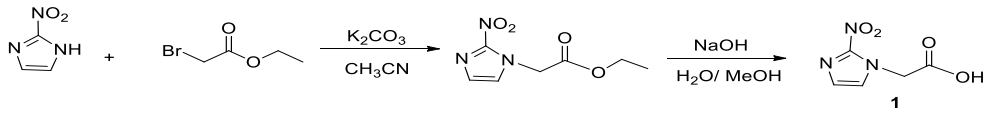
The precursor, 1-(2,3-diacetyl-5-tosyl-α-D-arabinofuranosyl)-2-nitroimidazole,the reference standard,1-(5-fluoro-5-deoxy-α-D-arabinofuranosyl)-2-nitroimidazole ([$^{19}$F]-FAZA), and the chelator, (DOTA-NHS) were purchased from Futurechem (Seoul, Korea).All other reagents were purchased from Sigma-Aldrich (Sao Paulo, Brazil) and were used as supplied without further purification.$^{1}$H-NMR and ESI-MS spectra were recorded at 400 MHz on Bruker Advance DRX 400 spectrometer and Bruker DaltonicsUltrOTOF-Q-II -ESI-Qq-TOF mass spectrometer, respectively. The chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS), used as the internal standard, by placing the multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet doublet, m = multiplet), the coupling constant (J), given in Hertz (Hz), and the number of hydrogens deduced from the relative integral. Activity measurements were performed either in a Dose Calibrator, Capintec CRC-5R.$^{68}Ge/^{68}Ga$-generator was obtained from Cyclotron Co (Obninsk, Russia). PET imaging was performed using an Albira μPET/SPECT/CT imaging system (Bruker Corporation, Spain).

### 2.1 Synthesis of Glypeptide-2-Nitroimidazole

#### 2.1.1 Synthesis of 2-nitroimidazole acetic acid

2-Bromoethyl acetate (BEA; 60 μL; 0.46 mmol) was added to a solution of 2-nitroimidazole (2-NI; 105 mg; 0.46 mmol) and potassium carbonate (100 mg, 0.69 mmol) in dry acetonitrile (10 mL) (Scheme 1). The mixture was reacted with constant stirring for 24 h at room temperature, and the resulting precipitate was filtered and washed with acetone. Evaporation of the organic filtrate under reduced pressure yielded a yellow oil (80.5mg). Analytical TLC revealed a product in one spot; *Rf* 0.66 (EtOAc: Hex 7:3). The purified oil of nitroimidazole ethyl acetate (80.5 mg; 0.43 mmol) was mixed into a solution of NaOH (4 M;125 μL; 0.43 mmol), water (0.5 mL), and MeOH (0.5 mL). The resulting solution was stirred at room temperature until no ester derivative was evident by TLC (~ 4 hr), at which point a cation-exchange resin (H+, Bio-Rad, 4 g), which had been protonated by washing with $H_2SO_4$ (1M;

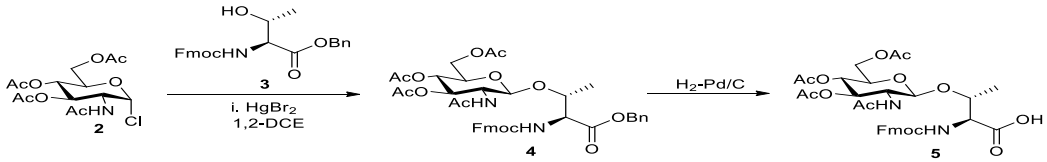
10 mL) and distilled water, was used to convert the basic solution to its acetic derivative (pH 2.5). Subsequent filtration and drying of the filtrate yielded a dark yellow paste (1.16 g). Chromatography of crude product on silica gel (eluent, EtOAc : Hex 7:3) yielded a white crystal: *Rf* 0.30 (EtOAc : Hex 7:3).(92.2 mg, 93.7%)MS *m/z* observed 172 $[M+H]^+$,calculated 172. $^1$HNMR ($CDCl_3$):$\delta_H$ 7.55 (1H, d, *H*-imidazole), 7.17 (1H, d,*H*-imidazole), 5.15 (2H, s, $NCH_2COO$), 3.90 (1H,broad, COOH).




**Scheme 1: Synthesis of the 2-nitroimidazole acetic acid derivative.**

### 2.1.2 Synthesis of the glycoamino acid βGlcNAc-ThrOH

A mixture of 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl **2** (500 mg, 1.37 mmol) and N- (9-fluorenylmethoxycarbonyl) -L-threonine **3** (292.7 mg, 6.80 mmol) in 1,2-dichloroethane (10 mL) was treated with $HgBr_2$ (489.7 mg, 1.37 mmol). The mixture was stirred and heated under reflux at 95 ° C for 14 h and was accompanied by TLC (EtOAc : Hex 7: 3). The obtained amber mixture was concentrated and purified on a silica gel chromatographic column (EtOAc :Hex 7:3 v: v). A solution of N- (9-fluorenylmethoxycarbonyl) -(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl) -L-threonine **4** ( 56 mg, 0.073 mmol) previously washed with 0.5% EDTA solution in MeOH (2.5 mL) was treated with glacial AcOH (0.2 mL) and 10% Pd/C (20 mg). The mixture was stirred and kept under $H_2$ atmosphere (~1.5 atm) for 1 h. The mixture was sintered onto celite, concentrated and purified on a silica gel chromatographic column (AcOEt: Hexane 7: 3 v: v) until formation of **5**. Product **5** was obtained as an amorphous white solid (46 mg, 0.068 mmol, 65.3%). $\delta_H$ ($CDCl_3$, 400 MHz) 7.78 (2 H, d, J 7.3 Hz, CH Fmoc arom.H-1, H-1 '), 7.65 (2H, d, J = 6 Hz, CH-Fmocarom. H-4, H-4 '), 7.43-7.28 (4 H, m, Fmocarom. H-2, H- 2', H- ), 6.12 (1H, d, J 9.2Hz, NHThr), 6.03 (1H, d, J 9.2Hz, NHAc), 5.25 (1H, t, J2.3 9.6 Hz, H-3), 5.00 (1 H, t, J 3.49 Hz, H-4), 4.65 (1 H, d, J 1.2, 8.3 Hz, H -1), 4.47-4.35 (4H, m, CH2 Fmoc, 2CH Thr), 4.30 (1H, dd, J 6.8Hz, J 10.6Hz, H-6a), 4.20-4.00 (2H, m, Fmoc CH, H-6b), 3.85 (1H, dd, J1.2, 8.1 Hz, J2.3, 10.1 Hz, H-2) , 3.75 (1 H, m, H-5), 2.04; 1.98; 1.97; 1.93 (12H, 4s, 4x COCH3), 1.20 (3H, d, J = 6.3 Hz, CH3 Thr). ESI-MS: calculated for $C_{33}H_{39}N_2O_{13}$ [M + H] +: 671.2452, found: 671.2453.



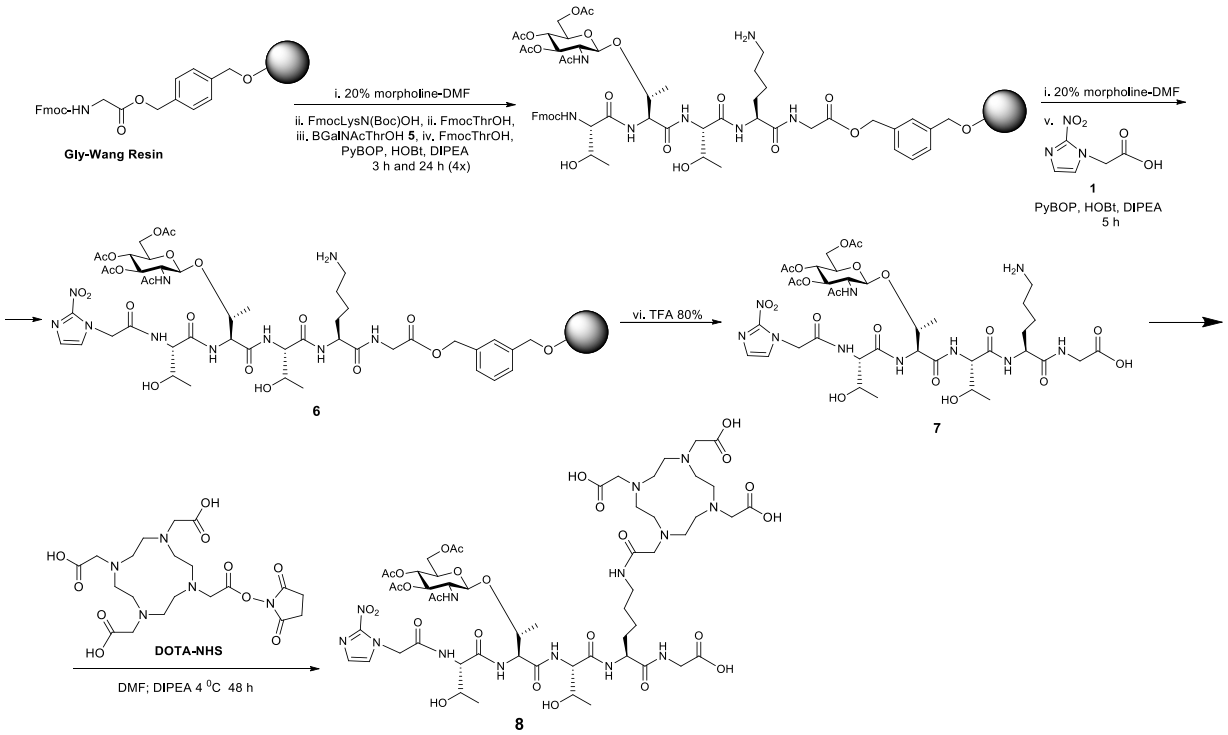

**Scheme 2: Synthesis of βGlcNAc-ThrOH5glycoamino acid.**

### 2.1.3 Solid-phase synthesis of the glycopeptide 2-nitroimidazole-Thr-Thr [βGalNAc] -Thr-Lys-GlyOH of the glycoamino acid βGlcNAc-ThrOHand conjugation to the chelator agent (DOTA-NHS)

Once intermediates **1** and **5** were obtained, glycopeptides **6** synthesis was then performed on solid support (Gly-Wang resin, 100 mg; 0.065 mmol) by deprotection reactions (20%

morpholine-DMF; 2 mL) and coupling of amino acid FmocLysN (Boc) OH (60.9 mg, 0.13 mmol) and FmocThrOH (51.67 mg; 0.13 mmol), βGlcNAc-ThrOH **5** glycoamino acid (27.2 mg; 0.04 mmol), and 2-nitroimidazole acetic acid **1** (6.84 mg; 0.04 mmol) in the presence of the PyBOP coupling reagents, HOBt (1-hydroxybenzotriazole) and DIPEA (N, N - Disopropylethylamine) in DMF. Next, cleavage of the glycopeptide 6 in the presence of 80% aqueous TFA solution provided the final glycopeptide **7**. (4.61 mg, 0.0054 mmol, 14%). $\delta_H$ ($D_2O$, 400 MHz),7.55 (1H, d, *H*-imidazole), 7.17 (1H, d, *H*-imidazole), 6.03 (1H, d, J 9.2Hz, NHAc), 5.25 (1H, t, J2.3 9.6 Hz, H-3), 5.15 (2H, s, NC*H*$_2$COO),5.11(2 H, s, $NH_2$Lys)5.00 (1 H, t, J 3.49 Hz, H-4),4.42 (1 H, d, J 1.2, 8.1 Hz, H-1), 4.40-4.25 (5 H, m, βCHThr), 4.20-4 (5 H, m, $CH_2$Gly, H-6a, H-6b, H-5), 3.50 (1 H, t, J 9) (1H, t, J = 9.8 Hz, H-4), 3.25 (1 H, m, H-2), 1.87 (3H, s, NHAc), 1.20-1.00 (15H, m, 5x $CH_3$Thr). ESI-MS: calculated for $C_{39}H_{59}N_{10}O_{20}$ $[M + H]^+$: 987.3907, found: 987.3907

Subsequently, the glycopeptides **7**, was submitted to the conjugation reaction with the macrocyclic chelator DOTA-NHS (Scheme 3). Thus, a mixture of **7** (2.6 mg, 2.63 μmol) in 300 μL DMF, DOTA-NHS (4.1 mg; 5.26 μmol) in 100 μL DMF and DIPEA (220 μL; 12.54 μmol) was stirred at 4 $^{o}$C for 48 h. The final product was concentrated and purified on a silica gel chromatographic column (AcOEt: Hexane 7: 3 v: v) until formation of the final product.(2.61 mg, 0.0041mmol, 11.1%). $\delta_H$ ($D_2O$, 400 MHz),9.15 (3H, s, COOH-DOTA), 7.63 (1H, d, *H*-imidazole), 7.19 (1H, d, *H*-imidazole), 6.03 (1H, d, J 9.2Hz, NHAc), 5.25 (1H, t, J2.3 9.6 Hz, H-3),5.35 (2H, s, NC*H*$_2$COO),5.23 (1 H, t, J 3.49 Hz, H-4),5.17 (2 H, s, $NH_2$Lys),4.62 (1 H, d, J 1.2, 8.1 Hz, H-1), 4.49-4.23 (5 H, m, βCHThr), 4.27-4.01(5 H, m, $CH_2$Gly, H-6a, H-6b, H-5), 3.56 (1 H, t, J 9), 3.33 (8 H, s, $NCH_2CO$), (1H, t, J = 9.8 Hz, H-4), 3.24 (1 H, m, H-2), 2.15 (16 H, s, $NCH_2CH_2$), 1.89 (3H, s, NHAc), 1.28-1.07 (15H, m, 5x $CH_3$Thr). ESI-MS: calculated for $C_{55}H_{85}N_{14}O_{27}$ $[M + H]^+$: 1373.5708, found: 1373.5709




**Scheme 4: Synthesis Glycopeptide-2-Nitroimidazole.**

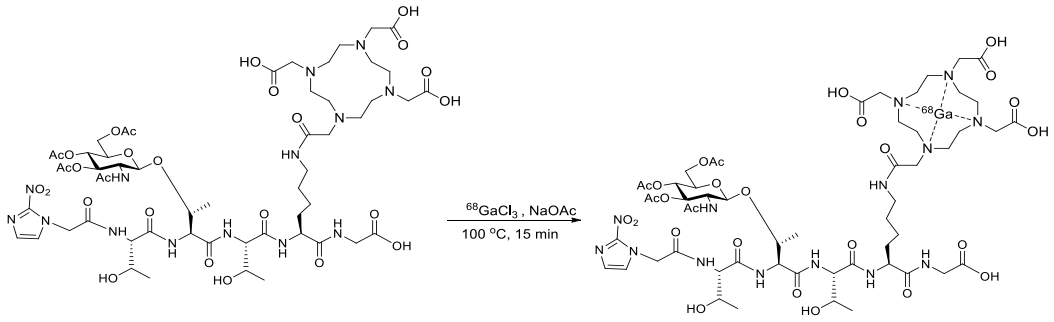
## 2.2 Radiolabelling of [$^{68}$Ga]Glycopeptide-2-Nitroimidazole

### 2.2.1 $^{68}$Ga and $^{68}$Ge / $^{68}$Ga generator

The $^{68}GaCl_3$ solution, 925 mBq (25 mCi), eluted from the generator with 10 mL 0.1M HCL solution, was concentrated and purified by the method of Zhernosekov et al. [23]. This $^{68}$Ga solution concentrated and purified was used in the labeling studies. Approximately 370 mBq (10 mCi) of activity was used in each reaction.

### 2.2.2 Radiosynthesis

The study was performed by adding aliquots of compound **8** (DOTA-Glycopeptide; 1 mg/mL) into 1 mL fractions of 1M Sodium Acetate solution (pH 4.5), subsequently added to 400 µL of the $^{68}GaCl_3$ solution. All experiments were run for 15 minutes in a shaking heating block. A factorial experimental design $2^2$ was performed to determine the influence of the reaction temperature (70 and 100 $^{o}$C) and the amount of glycopeptide (20 and 30 nmol) on the yield of the labeling. Purification of the compound was by reverse phase chromatography with Plus C-18 cartridges, primes with 5 mL of Ethanol and 10 mL of $H_20$.The reaction mixture was passed through the cartridge obtaining quantitative retention of the labeled glycopeptide and passing through the cartridge, free $^{68}$Ga. After washing the cartridge with 5 mL of water, the final compound was recovered with 400 µL of Ethanol (Scheme 5)




**Scheme 5: Radiosynthesis of [$^{68}$Ga]Glycopeptide-2-Nitroimidazole**

### 2.2.3 Quality control

After the radiosynthesis; the product identity, the chemical and radiochemical purity of the final formulation were determined by HPLC. HPLC analysis conditions were: ZORBAX Eclipse Plus C18 Analytical 4.6 x 250 mm (5 µm) column; Mobile phase: $H_2O$ (0.1% TFA) (A) and Acetonitrile (B) 0-100% B for 12 min and 100% B for 30 min, UV 280 nm. The radiochemical yield was calculated for each of the steps of radiosynthesis from the initial time of the corresponding reaction. Radiochemical purity was defined as the percentage associated with the product of interest in the analysis profile by the radioactivity detector and the chemical purity was assessed through the analysis profile by the UV detector at 280 nm.

## 2.3 Physicochemical Evaluationof [$^{68}$Ga]Glycopeptide-2-Nitroimidazole

### 2.3.1 Stability in physiological saline (0.9% NaCl)

Under best synthesis conditions, the stability of the compound was determined at room temperature in 0.1M PBS and pH = 7.2. The samples had activity of 3.7 MBq (1 mCi) in a final volume of 1 mL. The stability of the compound was monitored for 4 hours after labelling and the radiochemical purity was assessed by HPLC using chromatographic conditions described in 2.2.3.

### 2.3.2 DTPA challenge

[$^{68}$Ga]-Glypeptide-2-Nitroimidazole was incubated with excess of diethylenetriamine pentaacetic acid (DTPA) in aqueous solution (100 molar-excess) at 37 °C and radiochemical purity of the gallium complex was assessed by HPLC using chromatographic conditions described in 2.2.3 for up to 2 hours.

### 2.3.3 Lipophilicity

Lipophilicity was studied through the apparent partition coefficient between 1-octanol and water. In a centrifuge tube, containing 2 mL of each phase, 0.1 mL of the [$^{68}$Ga]-complex in solution were added, and the mixture was shaken on a Vortex mixer and finally centrifuged at 5000 rpm for 5 min. Three samples (0.2 mL) from each layer were counted in the dose calibrator, Carpintec. The partition coefficient was calculated as the mean value of each µCi/mL of 1-octanol layer divided by that of the water. Lipophilicity was expressed as log P. Lipophilicity of [$^{18}$F]FAZA was determined using the same methodology.

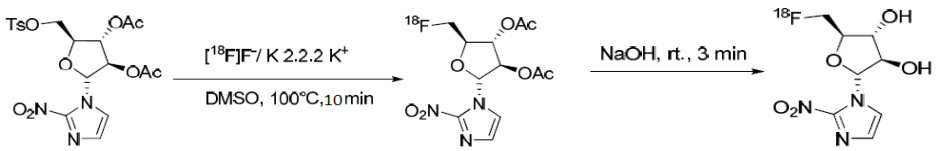
## 2.4 Synthesis of [$^{18}$F]FAZA

### 2.4.1 Production of [$^{18}$F]Fluoride

[$^{18}$F]Fluoride was produced via the $^{18}O(p,n)^{18}F$ nuclear reaction from [$^{18}$O]$H_2O$ (Rotem Industries Ltd, Hyox oxygen-18 enriched water, min. 98%) on an 18 MeV Cyclotron (Advanced Cyclotron Systems Inc., IBA, Belgium).Cyclotron-produced [$^{18}$F]Fluoride was trapped on a Waters SepPak® light QMA anion exchange cartridge, prime with 10 mL of $K_2C0_3$0.5 M and 20 mL of $H_20$.

### 2.4.2 Manual synthesis of [$^{18}$F]FAZA

The synthesis of [$^{18}$F]FAZA was performed manually, as described in the scheme 6.




**Scheme 6: Radiosynthesis of[$^{18}$F]FAZA**

We modified the reaction conditions by reference to those published previously [24,25]. Aminopolyether 2.2.2 (Kryptofix 222) (10 mg, 26 µmol),$K_2CO_3$ (13 µmol), n.c.a. [$^{18}$F]fluoride (2000–3000 MBq and acetonitrile (1 mL) were mixed in a 5 mL reaction vial (Whaton, United States). The water was evaporated under a stream of nitrogen (heating block 100 °C). The reaction mixture was co-evaporated with acetonitrile (1mL) three times. This

was followed by the addition of a solution of 1-α-D-(2,3-diacetyl-5-tosyloxy-arabinofuranosyl)-2-nitroimidazole (10 mg, 21μmol) in DMSO and the mixture was heated for 10min. The mixture was diluted to a final volume of 20 mL with water and loaded on a Sep-Pak C18 cartridge (Waters), preconditioned with each 5 mL ethanol and water. The cartridge was washed with 2 mL water and then eluted with 1mL ethanol. Ethanol was evaporated and acetylated [$^{18}$F]FAZA, so obtained, was hydrolyzed using 0.1 M NaOH (1 mL) for 5 min at 30 °C. The reaction mixture was neutralized by adding 0.1 MHCl (1 mL). Radiochemical purity was determined by HPLC: Stationary phase: Agilent C18, 4.6 mm x 250 mm, 5 μ, 100 μ. Mobile Phase: Solvent (A) Water Milli Q + 0.1% TFA, Solvent (B) 100% Acetonitrile. UV, 300 nm. Gradient: 30% to 100% Acetonitrile in 20 min. Flow: 1mL / min.

### 2.4 Biological Evaluation of [$^{18}$F]FAZA

#### 2.4.1 Cell culture

MKN45 cells were cultured in RPMI medium (Gibco, Life technologies, MD, USA) supplemented with 10% fetal bovine serum (Gibco, Life technologies, MD, USA) and 50 μg/mL gentamicin (Gibco, Life technologies, USA). Mycoplasma contamination was excluded using the Lonza micloplasm detection kit [26].

#### 2.4.2 In vivo evaluation of [$^{18}$F]FAZA uptake

For *in vivo* studies, Balb/c Nude mice were inoculated subcutaneously with 1x10$^6$ MKN45 cells. When the tumor reached about 300 mm$^3$ the animals were injected intravenously with 3.7MBq of [$^{18}$F]FAZA diluted in saline (0.9% NaCl). Two hours after the injection the animals were anesthetized with isoflurane by inhalation (initial concentration 0.5% - general anesthesia 1 to 1.87%), placed in the scanning chamber and analyzed in the μPET/CT.

## 3. RESULTS AND DISCUSSION

### 3.1Chemical Synthesis

Initially, the 2-nitroimidazole acetic acid derivative was obtained by the nucleophilic addition reaction of ethyl-2-bromoacetate to 2-nitroimidazole in the presence of $K_2CO_3$, followed by cleavage of the ethyl ester by the use of NaOH. The reaction resulted in the formation of 92.2 mg of the product and a yield of 93.7% the result was confirmed by $^1$H-NMR and ESI-MS

Next, the synthesis of the glycoamino acid βGlcNAc-ThrOH **5** was performed by glycosylation reaction of the glycosidic donor αGlcNAcCl **2** with the amino acid FmocThrOBn**3**, using the 1,2-dichloroethane as solvent and the $HgBr_2$ as catalyst, reaction mixture was maintained at reflux for 14 h (Campo, 2007), yielding 1.45 g of compound 4 as a white solid and a reaction yield of 40.6%. Identification of the βGlcNAc-FmocThrOBn **4** block was performed based on the coupling constants of the anomeric hydrogens in the $^1$H-NMR spectrum and showed characteristic signals as in δ 4.60 (1H, d, J1.2, 8.1, axial).The final step of preparation of the βGlcNAc-FmocThrOBn **4** building block for subsequent peptide chain coupling involved the removal of the O-benzyl ester group of **4** by means of the classical catalytic hydrogenation reaction (10% Pd-C/$H_2$). Considering the contamination of

the 10% Pd-C catalyst by the $HgBr_2$ promoter, existing in residual amounts in the starting materials, a solution of 4 in ethyl acetate was carried out followed by washing with 0.5% EDTA solution prior to the hydrogenation reaction, with the purpose of sequestering possibly present metallic ions. Next, the reaction mixture was subjected to stirring at room temperature under $H_2$ (~1.5 atm) for 1 h, with monitoring by TLC due to the possibility of removal of the N-protecting group Fmoc in prolonged periods of reaction. Thus, the free carboxyl group product 5 was obtained in 65.3% yield and confirmed by $^1$H-NMR and ESI-MS.

In order to obtain glycopeptide 7 the solid phase peptide synthesis method was used. It has a number of advantages over the methods in solution; (I) the reactions can be carried out in the presence of a large excess of the reactants, forcing the product to be obtained; (II) the excess of the reactants and by-products of the reaction can be removed by successive washes of the resin; (III) product losses are minimal because it remains bound to the resin throughout the synthesis; (IV) allows the preparation of analogous compounds in series, as in combinatorial synthesis [27]. The coupling of the amino acids and intermediates 1 and 5 for elongation of the peptide chain were performed in DMF in the presence of the coupling reagents PyBOPand HOBt, used to avoid possible enantiomerization reactions, and the base DIPEA (diisopropyl ethylamine). The reactions were monitored from resin samples by the Kaiser test, which is able to evaluate the existence of a free amino group, and in a UV spectrophotometer (290 nm), by the liberation of dibenzofulvene. In all cases the intense blue staining confirmed the presence of free amino group bound to the resin.

The coupling rates obtained varied from 0.80 mmol/g to 0.90 mmol/g for the amino acid FmocThrOH, 0.70 mmol/g for the amino acid Fmoc-Lys(Boc)OH, 0.65 mmol/g for the glycoamino acid βGlcNAc-ThrOH **5** and 0.75 mmol/g for 2-nitroimidazole acetic acid **1**. Next, cleavage of glycopeptide **6** in the presence of 80 % aqueous TFA solution gave a mass of 4.61 mg, and a yield of 14% of the final glycopeptide **7**. The final time of the synthesis was seven days and the ESI-MS analyzes confirmed the glycopeptide **7**, and the corresponding characteristic adduct $[M+H]^+$ 987.39 was verified in the positive mode. The $^1$H-NMR spectrum of glycopeptide **7** also showed characteristic signals for the Hs of the nitroimidazole group at δ 7.4, the H-1 of the βGlcNAc sugar at δ 5.0, and the OAc groups of, $CH_2$ Lys and $CH_3$Thr in the region between δ 2.08-1.09. Subsequently, the glycopeptides **7** was subjected to the conjugation reaction with the macrocyclic chelator DOTA. The reaction resulted in the formation of 2.61 mg of the product and a yield of 11.1% thereof, the ESI-MS spectrum showed the adduct $[M+H]^+$ 1373.57, consistent with the mass of the conjugated glycopeptide, confirming the final product formation result. Further, to prove the purity of the compound an HPLC analysis was performed (Figure 1), reporting a 98.7 % purity of chemistry ($R_T$ = 8.7 ± 0.2 min).

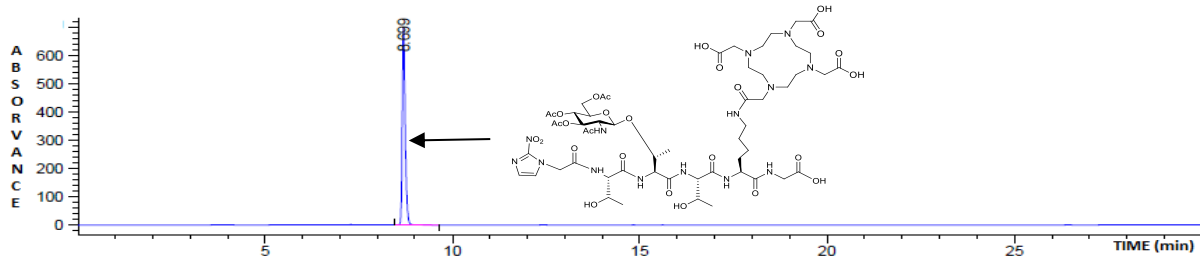



**Figure 1: HPLC analysis UV chromatogram of reference standard, glycopeptide 7 with the chelator agent DOTA-NHS (1 µg/mL, retention time 8.7 min).**

### 3.1 [$^{68}$Ga]Glycopeptide-2-Nitroimidazole

Currently, it is not possible to directly use the $^{68}$Ga eluate in the preparation of radiopharmaceuticals. This is mainly due to the appreciable content of $^{68}$Ge in the eluate, the large volume of the solution eluted (~ 10ml of HCl 0.1 M) and the high concentration of HCl in it. Also impurities of metals, such as $Zn^{2+}$, generated from decay of $^{68}$Ga and others from the material ($Ti^{4+}$, $Fe^{3+}$, $Al^{3+}$) of the column are present [28].To solve this problem, a method of purification and concentration based on the literature data has been developed, which indicates that with the increase of the content of acetone in acid solution, there is a decrease in the affinity of the gallium by a cationic resin [23]. A microchromatographic column containing a cation resin was then used to perform $^{68}$Ga purification after elution of the $^{68}$Ge/$^{68}$Ga generator. The recovery of $^{68}Ga^{3+}$ occurred in an acetone/HCl solution and showed that this method is simple, fast and chemically efficient in removing the following impurities: Ge, Fe, Zn and Ti and pre-concentration, since the initial volume of the eluate was 10 mL of 0.1 M and passed to 400 μL of the Acetone/HCl (98/2)% mixture.

Macrocyclic chelates, due to the high stability of the complexes formed to the acyclic, are excellent compounds, widely used in radiopharmacy for the complexation of $^{68}$Ga. However, several compounds derived from DOTA such as $^{68}$Ga-DOTA-peptides, require heating at elevated temperatures for their formation [29]. Based on this fact, the reaction temperature was selected as one of the independent variables of the design. Furthermore, with the use of 10 nmol DOTA-peptides, the labeling does not occur for the temperature conditions selected for this study (observed experimentally), so that amounts of the glycopeptide slightly above were chosen to evaluate their influence on the radiochemical yield, in order to observe if for these levels of the variable there is a significant influence. The results of the experimental design are shown in table 1.

**Table 1: Effect of Temperature and the Glycopeptide on radiochemical yield for [$^{68}$Ga]Glycopeptide-2-Nitroimidazole**

| Entry | Temperature ($^{o}$C) | n(Glycopeptide) (nmol) | Radiochemical yield (%) |
|---|---|---|---|
| 1 | 70 | 20 | 39.3 |
| 2 | 100 | 20 | 92.9 |
| 3 | 70 | 30 | 41.5 |
| 4 | 100 | 30 | 93.8 |

The only significant variable is the reaction temperature, however, the amount of the glycopeptide and the interaction between the two variables were not significant. The labeling yield is favored with the highest temperature value. This is possible because at 100 °C the chelating deprotonation (DOTA) is favored, which facilitates complex formation. Furthermore, in the acidic conditions of the reaction mixture (pH 4.5), Gallium is present as $Ga(H_2O)_6$ and with the increase in temperature the speed of exchange of water molecules involved in the coordination sphere is favored the cation complexation. The fact that the amount of glycopeptide is not a significant variable in the region studied can be given since the $^{68}$Ga used in the labeling is present in excess by several orders of magnitude so that a variation of n does not lead to changes labeling yield. The radiochemical purity was 98.3%, the total time of synthesis was 38 minutes and the radiochemical yield was 57.3%. These results were determined by HPLC, the retention time of the compound being 8.2 ± 0.2 min minutes, as shown in Figure 2.

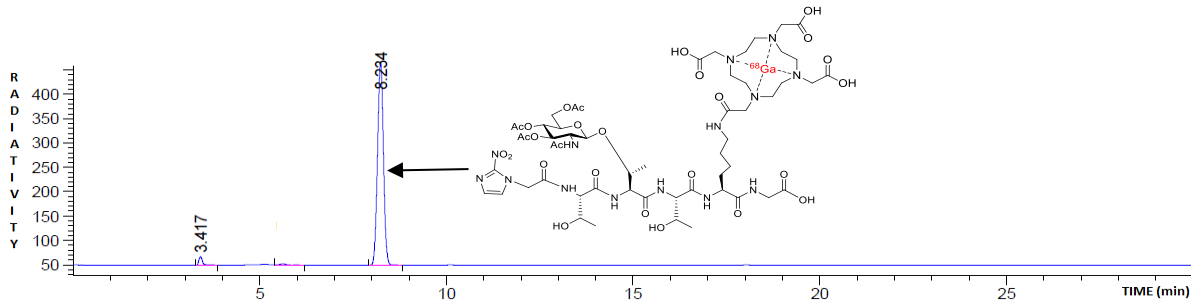



**Figure 2: HPLC analysis radioactivity chromatogram of the [$^{68}$Ga]Glycopeptide-2-Nitroimidazole(50 µCi, retention time 8.2 min)**

Evaluating the stability of the labeled compounds is one of the most essential parameters for determining whether a drug has the potential to be used for in vivo applications or not. In this work the stability of the new radiopharmaceutical was evaluated in a sterile saline solution for a period of 4 hours. The compound was stable for at least 4 h in labelling milieu and found to be over 90%.The acyclic ligand DTPA gallium complexes have high affinity but poor kinetic stability. Derivatizing the carbon backbone of DTPA can increase the stability of the $^{68}$Ga complexes [30], thus determining whether a $^{68}$Ga-labeled radiopharmaceutical presents trans-chelation with DTPA is one of the most important parameters to determine future applications of it. Incubation of [$^{68}$Ga]Glycopeptide-2-Nitroimidazole with a 100-molar excess of DTPA showed high stability(100%) and no trans-chelation of the gallium. Both stability results were positive for *in vivo* applications of the new radiopharmaceutical for tumor hypoxia imaging.

The partition coefficient between 1-octanol and water of the [$^{68}$Ga]-complex was measured in order to assess its lipophilicity. A logP of −1.65±0.05 was obtained, this value is in agreement with the proposed structure of complexes since incorporation of a DOTA and glycopeptides units increases hydrophilicity of molecule. A log P of −0.73±0.03 was obtained for [$^{18}$F]FAZA using the same methodology. [$^{68}$Ga]Glicopeptídeo-2-Nitroimidazole is significantly more hydrophilic than [$^{18}$F]FAZA and this is a clear advantage considering that slow washout from normoxic tissues caused by excessive lipophilicity, is considered a serious drawback of this radiopharmaceutical.

### 3.3 [$^{18}$F]FAZA

Next, we proceeded to obtain the [$^{18}$F]FAZA radiopharmaceutical, widely used in other countries for the detection of hypoxia, in order to serve as a standard of comparison with the new radiopharmaceutical proposed in this paper. At the reaction temperature of 100 °C, with DMSO we obtained an $^{18}$F labeling yield of 86.9%. However, with MeCN the yield obtained was slightly lower, of 77.1%. Therefore, we decided to use DMSO as the reaction solvent in this study. We also examined the effect of the reaction temperature at 80, 100 and 120 ° C in DMSO and the best result was at 100 ° C. This labeling yield decreased to 75.1% when the precursor was reduced by half 5.1 µmol of precursor (Precursor/Base ratio: 0.5), so we concluded that with the decrease in the P/B ratio a precursor decomposition occurs, labeling yield was lower. From these results, we decided to use the reaction condition of 10.2 µmol of precursor, reaction temperature of 100 °C, for 10 min and DMSO as reaction solvent for the routine production of [$^{18}$F]FAZA, which gave the best result of 86.9%.

The labeling of the compound was confirmed after HPLC analysis comparing the result with the retention time of the standard compound (Figure 3), ($R_T$ = 5.88 ± 0.2 min) and the labeled product (Figure 4), ($R_T$ = 5.91 ± 0.2 min), the radiochemical purity analyzed by HPLC showed a final result of 99.2%. The final radiochemical yield of the reaction was 40.1 %, the total time of the synthesis was 54.2 minutes, better results compared to the results in the literature (QualiHayashi, [28,29]2011; Reischl, 2005) so a new route of manual radiosynthesis was established for future comparison with the new radiopharmaceutical.

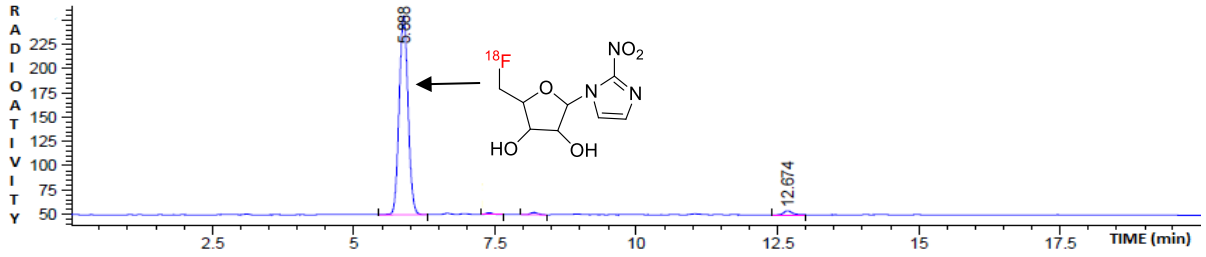



**Figure 3: HPLC analysis radioactivity chromatogram of the [$^{18}$F]FAZA(50 µCi, retention time 5.88 min)**

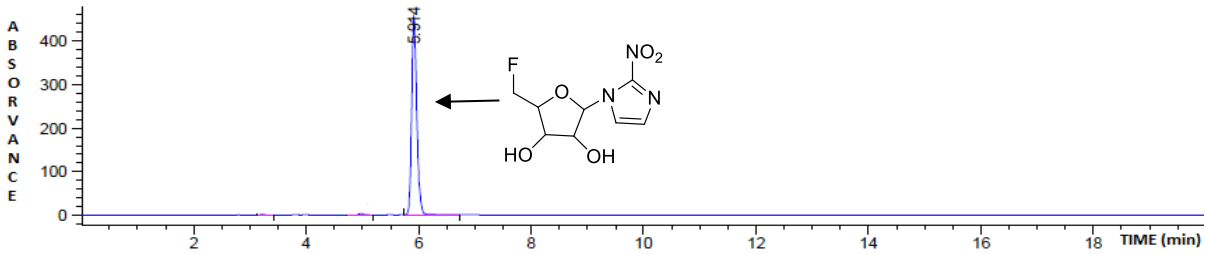



**Figure 4: HPLC analysis UV chromatogram of reference standard, [$^{19}$F]FAZA(1 µg/mL, retention time 5.91 min).**

Stomach Cancer (or Gastric Cancer) is the growth of abnormal cells in the organ of this digestive system and can occur anywhere in its extension. As cancer progresses, these abnormal cells gradually replace the normal tissue of the organ, spreading to other layers of the stomach and can affect neighboring organs (metastasis). According to data from the National Cancer Institute, 21,300 new cases will be diagnosed this year here in Brazil, with 13,540 men and 7,750 women and in relation to mortality, for both sexes, it becomes the third cause in world-wide level, with 723 thousand deaths, 8,8% of the total (Silva,[1] 2016). Currently, the standard diagnostic strategy is surgery, followed by biopsy, and the development and validation of noninvasive techniques is desirable. As [$^{18}$F]FAZA was obtained with optimal radiochemical purity, it was also used for the detection of hypoxia in a tumor model of gastric cancer. The final result (Figure 5) was the capture of the radiopharmaceutical at the tumor site so that we can take this tumor model as a comparison in the future new radiopharmaceutical, [$^{68}$Ga]Glycopeptide-2-Nitroimidazole.

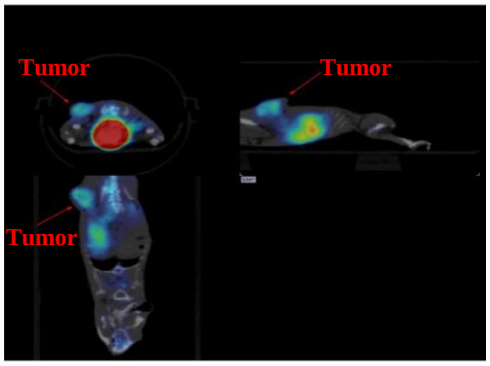



**Figure 5: Use of [$^{18}$F]FAZA in a gastric tumor model, MKN45**

## 4. CONCLUSIONS

This paper presents the development of one novel 2-nitroimidazole derivative and the corresponding [$^{68}$Ga]-complex, [$^{68}$Ga]Glycopeptide-2-Nitroimidazole, as potential radiopharmaceutical for hypoxia PET imaging. A new glycopeptide was effectively obtained by combined in solution and solid-phase methods in good yield and structural characterization was made by NMR spectroscopy and mass spectrometry. Labelling was achieved with highs radiochemical purities for [$^{18}$F]FAZA and a new [$^{68}$Ga]Glycopeptide and a complex resulted stable in labelling milieu and did not show trans-chelation in the presence of the challenging ligand DTPA. Gallium compound was significantly less lipophilic than the reference compound [$^{18}$F]FAZA make this compound promising candidate for further evaluation as potential hypoxia imaging agent and the image of [$^{18}$F]FAZA in a tumor model of gastric cancer was satisfactorily obtained. The new radiopharmaceutical potential for tumor hypoxia image will be tested soon *in vitro* and *in vivo* studies and with the same conditions their results will be compared with the established radiopharmaceutical [$^{18}$F]FAZA.

## ACKNOWLEDGMENTS

Authors gratefully acknowledge financial support from the Brazilian agencies Fundação de Amparo à Pesquisa do Estado de São Paulo [FAPESP] and Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq].

## REFERENCES

1. "Estimativa 2018: Incidência de Câncer no Brasil. Ministério da Saúde,Instituto Nacional de Câncer", http://www1.inca.gov.br/estimativa/2018/estimativa-2018.pdf (2018).
2. Vaupel P, Mayer A,"Hypoxia in cancer: significance and impact on clinical outcome", *Cancer Metastasis Rev*, **26(2)**, pp. 225-239 (2007).
3. Semenza GL, "Hypoxia, clonal selection, and the role of HIF-1 in tumor progression", *Crit Rev Biochem Mol Biol*, **35**, pp. 71–103 (2000).

4. Rofstad EK, Sundfor K, "Hypoxia-induced treatment failure in advanced squamous cell carcinoma of the uterine cervix is primarily due to hypoxia-induced radiation resistance rather than hypoxia-induced metastasis",*Br J Cancer*, **83**, pp. 354–9 (2000).
5. Tredan O, Galmarini CM, Patel K, Tannock IF,"Drug resistance and the solid tumor microenvironment",*J Natl Cancer Inst*, **99**, pp. 1441–54 (2007).
6. Gottesman MM, Fojo T, Bates SE, "Multidrug resistance in cancer: role of ATP dependent transporters", *Nat Rev Cancer*, **2**, pp. 48–58(2002).
7. Vaupel P, Hoeckel M, Mayer A,"Detection and characterization of tumor hypoxia using $pO_2$ histography", *Antioxid Redox Signal*, **9**, pp. 1221–35 (2007).
8. Ljungkvist ASE, BussinkJ, Kaanders J, Van der Kogel AJ,"Dynamics of tumor hypoxia measured with bioreductive hypoxic cell markers",*Radiat Res*, **167**, pp. 127–45 (2007).
9. Krohn KA, Link JM, Mason RP, "Molecular imaging of hypoxia",*J Nucl Med*, **49**, pp. 129–48 (2008).
10. Fleming IN, Manavaki R, Blower, "Imaging tumour hypoxia with positron emission tomography",*Br J Cancer*, **112**, pp. 238–50 (2015).
11. Lopci E, Grassi I, "PET radiopharmaceuticals for imaging of tumor hypoxia: a review of the evidence",*Am J Nucl Med Mol Imaging*, **4**, pp. 365–84 (2014).
12. Masaki Y, "Imaging massspectrometry revealed the accumulation characteristics of the 2-nitroimidazolebasedagent "pimonidazole" in hypoxia"*Sci Rep*, **5**, pp. 16802 (2015).
13. Masaki Y, Shimizu Y, "The accumulation nmechanism of the hypoxia imaging probe "FMISO" by imaging mass spectrometry:possible involvement of low-molecular metabolites"*PLoS One*, **11**, pp. 16-39 (2016).
14. Rajendran JG, "Tumor hypoxia imaging with [F-18]fluoromisonidazole positron emission tomography in head and neck cancer",*Clin Cancer Res*, **12**: pp. 5435–41 (2006).
15. Takasawa M, Moustafa RR, Baron JC,"Applications of nitroimidazole in vivo hypoxia imaging in ischemic stroke",*Stroke*, **39**, pp. 1629-37 (2008).
16. Okamoto S, Shiga T, Yasuda K ,"High reproducibility of tumor hypoxia evaluated by $^{18}$F-fluoromisonidazole PET for head and neck cancer", *J Nucl Med*, **54**, 201–7 (2013).
17. Sorger D, Patt M, Kumar P,Wiebe LI, Barthel H, Seese A, et al,"[$^{18}$F]Fluoroazomycin arabinofuranoside ($^{18}$FAZA) and [$^{18}$F]-Fluoromisonidazole ($^{18}$FMISO): a comparative study of their selective uptake in hypoxic cells and PET imaging in experimental rat tumours", *Nucl Med Biol*, **30**, pp. 317–26 (2003).
18. Tran L, Bol A, Labar D, Jordan B, Magat J, Mignion L, et al,"Hypoxia imaging with the nitroimidazole$^{18}$F-FAZA PET tracer: a comparison with OxyLite, EPR oximetry and $^{19}$F-MRI relaxometry"*Radiother Oncol*, **105**, pp. 29–35 (2012).
19. Lee ST, Scott AM, "Hypoxia positron emission tomography imaging with $^{18}$Ffluoromisonidazole", *Semin Nucl Med*, **37**, pp. 451–61 (2007).
20. Mukai T, Suwada J, Sano K, "Designed of Ga-DOTA-based bifunctional radiopharmaceuticals: two functional moieties can be conjugated to radiogallium-DOTA without reducing the complex stability",*Bioorg Med Chem*, **17**, pp. 4285-90 (2009).
21. Hoigebazar L,"Synthesis and characterization of nitroimidazole derivatives for $^{68}$Ga-Labeling and testing in tumor xenografted mice",*J Med Chem*, **53**, pp. 6378-85 (2010).
22. Hoigebazar L, Jeong JM, Hong MK, Kim YJ, Lee JY, Shetty D, et al. "Synthesis of 68GalabeledDOTA-nitroimidazole derivatives and their feasibilities as hypoxia imaging PET tracers", *Bioorg Med Chem*, **19**, pp. 2176-81 (2011).
23. Zhernosekov K, Filosofov D, et al, "Processing of generator-produced $^{68}$Ga for medical application", *J. Nucl. Med*, **48**, 1741–1748 (2007)
24. Reischl G, "Preparation of the hypoxia imaging PET tracer [$^{18}$F]FAZA: reaction parameters and automation", *Elsevier*, **69,** pp. 897-901 (2004)

25. Hayashi Kl, “High-yield automated synthesis of [18F]fluoroazomycin arabinoside ([$^{18}$F]FAZA) for hypoxia-specific tumor imaging”, *Elsevier*,**62,** pp. 1007-1013 (2011)
26. Tamura G, Sakata K, et al, “Inactivation of the E-cadherin gene in primary gastric carcinomas and gastric carcinoma cell lines”, *Japanese journal of cancer research,* **87**, pp.1153-1159 (1996).
27. Seeberger H, Haase W, “Solid-Phase Oligosaccharide Synthesis and Combinatorial Carbohydrate Libraries”, Chem. Rev, **100**, pp. 4389-4393 (2000).
28. Rösch F, “Past, present and future of $^{68}$Ge/$^{68}$Ga generators”. *Applied Radiation and Isotopes*, **76**, pp. 24–30 (2013).
29. Fukase K, “PET imaging of biomolecules using metal-DOTA complexes: a new collaborative challenge by chemists, biologists, and physicians for future diagnostics and exploration of in vivo dynamics”, *Org. Biomol. Chem*, **6**, pp. 815-828 (2008).
30. Wagner, SJ, “Gallium-68 labeling of albumin and albumin microspheres”, *J Nucl Med*, **20**, pp. 428-33 (1979)