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Preparation, Biodistribution of ^{99m}Tc-Azacytidine Complex as a Potential Agent for Radiotherapy

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ABSTRACT

Objectives: This study aimed at exploring the possibility of radiolabeling 5-Azacytidine with technetium-99m (^{99m}Tc) as a surrogate for Rhenium- 186/188 in order to explore the possibility of using the radiolabeled complex as a targeted radiotherapeutic agent. **Methods:** Radiolabeling of 5-Azacytidine with ^{99m}Tc *via* complexation reaction using stannous chloride dihydrate as a reducing agent. The biodistribution of the formed complex was then studied in tumour bearing mice. **Results:** Successful radiolabeling of 5-Azacytidine with ^{99m}Tc was achieved with radiolabeling yield 84.94%. The formed complex showed *in vitro* stability up to 60 min post labelling. The biodistribution study of the complex showed a Target /Non-target ratio of 2.27 at 2h post injection suggesting good localization of the complex. **Conclusion:** The radiolabeling of 5-Aza was successfully achieved with ^{99m}Tc. The biodistribution study in tumour bearing mice showed good localization of the radiolabeled complex at the tumour site, however further studies are needed for achieving better targeting of the complex.

Keywords: *5-Azacytidine; Biodistribution; Radiolabeling; Radiochemotherapy; ^{99m}Tc.*

INTRODUCTION

5-Azacytidine ((4-amino-1-β-D-ribo-furanosy1 -1,3,5 triazine-2-one), 5-Aza, is a pyrimidine nucleoside analogue that was chemically synthesized by Piskala*et.al* [1]. It was found to have a wide range of biological activity with the cytotoxic and antineoplastic effect as the major concerns[2,3]. 5-Aza is used mainly for the treatment of Acute Myeloid Leukaemia (AML) and Myelodysplastic Syndrome (MDS)[3,4]. The usual route of administration of this drug is *via* subcutaneous (s.c) or continuous (i.v) infusion due to severe nausea and vomiting caused by the i.v. bolus injection of 5-Aza[5-8]. The antitumor effect of 5-Aza is due two main mechanisms of action (**Figure 1** and **2**).

The first mechanism includes the phosphorylation and incorporation into the cellular RNA or DNA after its reduction as illustrated in **Figure 1**. This incorporation causes a disruption of the protein synthesis within the cell. The second mechanism involves the inhibition of DNA methyltransferase1 (DNMT1) *via* irreversible binding which ceases the enzyme progression along the DNA (**Figure 2**)[3,9,10]. DNMT1 methylate both cytosine residue and 5-Aza when incorporated into the DNA. The presence of 5-Aza will prevent the resolution of the covalent reaction intermediate (**Figure 2**). As a result, DNMT1 within the cell will be depleted[11].

Radiation therapy (XRT) is used for treatment of various types of cancers by utilizing the damaging effect of ionising radiation on the tumour cells. XRT can be achieved using an external beam radiation source, an approach that is limited only for patients with operable tumours. Also, XRT can be achieved using brachytherapy seeds which localizes the radioactive isotope near or within the tumour tissue in order to minimize the damage of surrounding tissues[12]. Rhenium-188/186 ($^{188/186}$Re) isotopes are suitable for

radiotherapeutic purposes as they emit β^- particles which causes cellular death. ^{186}Re have a half-life ($t_{1/2}$) of 90h and emits β^- particles with 1.07MeV energy. ^{188}Re ($t_{1/2}$ = 17 h) and also emits β^- particles with energy of 2.1 MeV. $^{188/186}$Re is the therapeutic match of Technitium-99m (^{99m}Tc) as they both lie in the same group of the periodic table (group VIIB) hence, they possess close chemical characteristics. ^{99m}Tc is a generator produced radionuclide with suitable half-life ($t_{1/2}$ = 6h) and suitable energy (140KeV) for nuclear medicine imaging technique [13,14]. In this study ^{99m}Tc was used as a surrogate for $^{188/186}$Re due to its ease of availability[13,14].

For radiotherapeutic purposes, radiolabeling of several available chemotherapeutic agents have been achieved by several researchers. Dimercaptosuccinic acid (DMSA) was radiolabeled with ^{188}Re by Garcia-Sallinas *et al*. and studied for cervical cancer therapy [15]. Amin *et al*. studied the possibility of using ^{125}I-Thioguanin for radiotherapy in Ehrlich ascites bearing mice[16]. Bayoumi *et al.* radiolabeled Cladribine with ^{125}I as apotential agent for cancer radiotherapy and imaging in solid tumour bearing mice[17]. ^{188}Re-Lanreotide (a somatostatin analogue) was prepared by Molina-Trinidad *et al.*and studied for the effective treatment of various tumours in mice[18].

Other researchers took a different approaches to combine the value of radiotherapy and chemotherapy together an approach known as radiochemotherapy. In 2005, Azhdarinia *et al.* prepared a hydrogel radiolabeled with ^{188}Re (a radiotherapeutic isotope) and loaded it with Cisplatin (a chemotherapeutic agent) for studying the radiochemotherapeutic effect in rats bearing solid tumour[12]. A liposomal formulation containing Doxorubicin (a chemotherapeutic agent) was radiolabeled with ^{186}Re and studied for its radiochemotherapeutic effects on Head and Neck Squamous Cell Carcinoma Xenograft Model by the efforts of Soundararajan *et al*[19]. Shih and co-workers explored the possibility of using ^{188}Re-Doxorubicin loaded in a micelle formulation for the treatment of hepatocellular carcinoma (HCC) [20].

In this work, radiolabeling of 5-Aza with ^{99m}Tc as a surrogate for $^{188/186}$Re was attempted to evaluate the possibility of its use as a radiotherapeutic agent or in a combination with 5-Aza for radiochemotherapeutic effect. In the following sections, the radiolabeling process and biodistribution are discussed in details.

MATERIALS AND METHODS

Chemicals and equipment

5-Azacytidine was purchased from Sigma-Aldrich Chemical Company and stored as instructed on the label. The chemicals and reagents used in this work were of reactive grade and bought from Sigma-Aldrich Chemical Company and Merck Company. Purged deoxygenated bi-distilled water was used in all experiments and procedures. Technetium-99m was eluted in the form of ^{99m}TcO$_4^-$ from ^{99}Mo/^{99m}Tc generator, Gentech, Turkey. A good-type NaI scintillation ɤ-Counter model scalar ratemeter SR7 (Nuclear Enterprises Ltd. USA). Whatmann paper no.1 was used for the paper chromatography (1cm x 13 cm strips).

Preparation of ^{99m}Tc-Aza complex

SnCl$_2$.2H$_2$Ostock solution was prepared as follows; 95 mg of SnCl$_2$.2H$_2$O was placed in a 5 ml volumetric stoppered flask and heated with 0.5 ml conc. HCl until complete dissolution. The volume was then completed using purged deoxygenated bi-distilled water. The prepared solution was diluted so that each 1ml of the solution contained 1.9mg of SnCl$_2$. 2H$_2$O equivalent to 1 mg Sn (II).

A freshly prepared aqueous solution of 5-Aza (1:1 w/v) was always used during this study. Different volumes of 5-Aza solution containing 200-1000 μg were placed in evacuated penicillin vials. Exactly 100 μl (200 MBq) of freshly eluted ^{99m}TcO$_4^-$ solution was then added to the vial. After that, different volumes of SnCl$_2$.2H$_2$O stock solution containing 200-2500μg of Sn(II) was added. The reactions were kept at ambient temperature (25 ± 5^0C) for up to 120 min.

Determination of the radiolabelling yield

Paper chromatography was used for the determination of the radiolabelling yield. Two strips of ascending Whatmann paper no. 1 were used for each procedure, on each strip, 2 drops of the reaction mixture were placed on the origin line, 2 cm far from the base. To determine percentage of free ^{99m}TcO$_4^-$, the developing solvent for one paper strip was acetone. The other paper normal saline was used to determine the percent of ^{99m}Tc-reduced hydrolysed species. At the end of the development process the strips were allowed to dry cut into 1cm pieces. The pieces were counted in well type ɤ-counter.

The % of ^{99m}Tc-Aza was calculated from the following equation:

% Radiolabeling yield = 100 - (% Free ^{99m}Tc + % ^{99m}Tc- reduced hydrolysed species)[21].

Biodistribution study

In accordance with the Egyptian Atomic Energy Authority (EAEA), Animal studies were executed and it was also asserted by Animal ethics committee. Animal models used in the *in vivo* experiment were tumour bearing Swiss albino mice of body mass range 25-30 g and were purchased from Helwan University, Egypt, were used in this study.
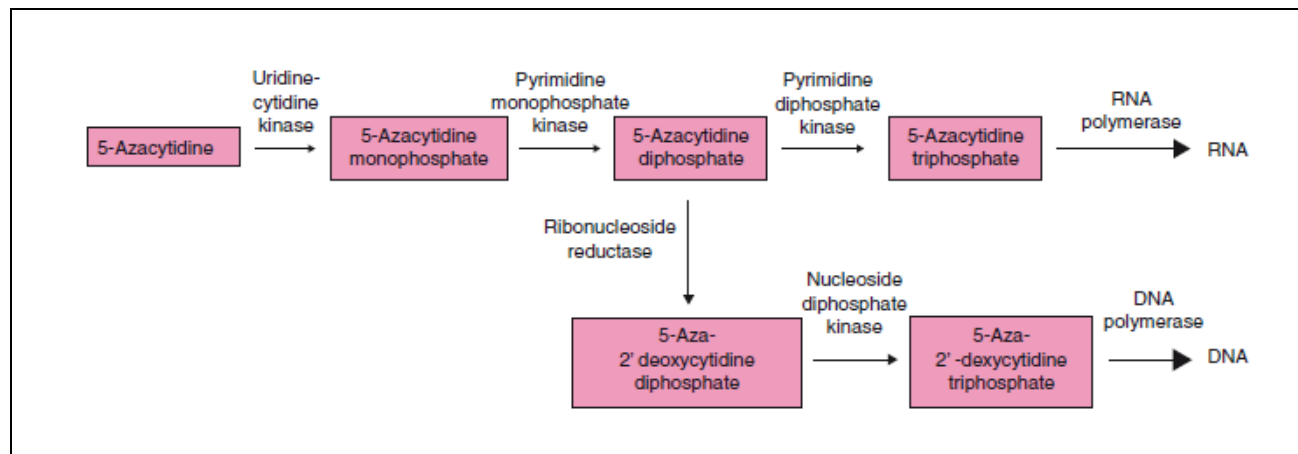
Figure 1. Schematic representation of 5-Aza incorporation into RNA and DNA.
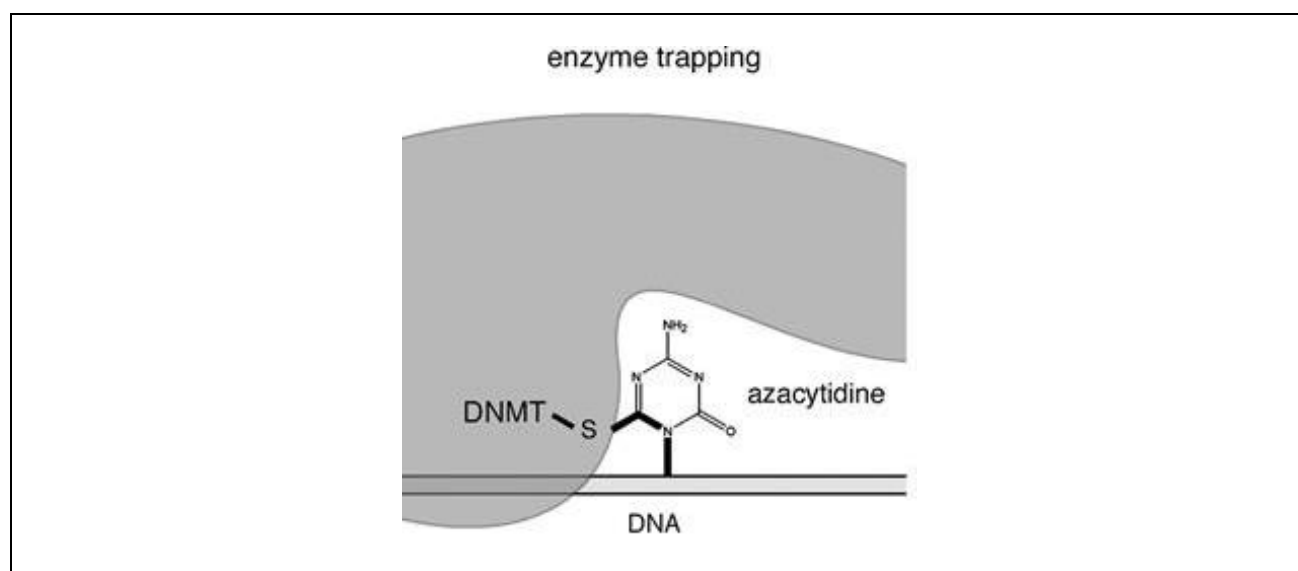

Figure 2. Schematic representation of the irreversible inhibition of DNMT1[11].

The animals were on a pellet diet and tap water and they were maintained in a cabin of a size that suits groups of three at ambient temperature with a 12 h light/dark cycle. Animal treatment was in accordance with the National Institute of Health Guide for Animal, as approved by the Institutional Animal Care and Use Committee (IACUC).

The Ehrlich ascites carcinoma (EAC) was withdrawn from the ascetic fluid of a mice bearing EAC (10 days old) under aseptic conditions and diluted using physiological sterile saline solution. The diluted cell line was mixed for 5 min by vortex shaker. To generate Ehrlich solid tumour, 200 µl of the diluted ascetic fluid was injected intramuscularly in the right lower limb of each mouse. The biodistribution study was conducted when a palpable solid mass was observed 10 days after inoculation. The mice were divided into 5 groups (n=3) and the radiolabeled complex was injected into the tail vein of each mouse. The animals were anaesthetize using chloroform then weighed and sacrificed at different time points following the administration of the complex (0.25, 0.5, 1, 2 and 4 h). All body organs were detached, washed with normal saline and left to dry then weighed. Blood specimen was collected *via* cardiac puncture. Blood, bone and muscle were assumed to be about 7, 10 and 40% of the total body mass of each mouse, respectively[22,23]. Well type γ-counter was used to count the activity of the collected organs/body fluid and the background. The percent-injected dose/gram organ or fluid (% ID/gm) was calculated in aggregates of three.
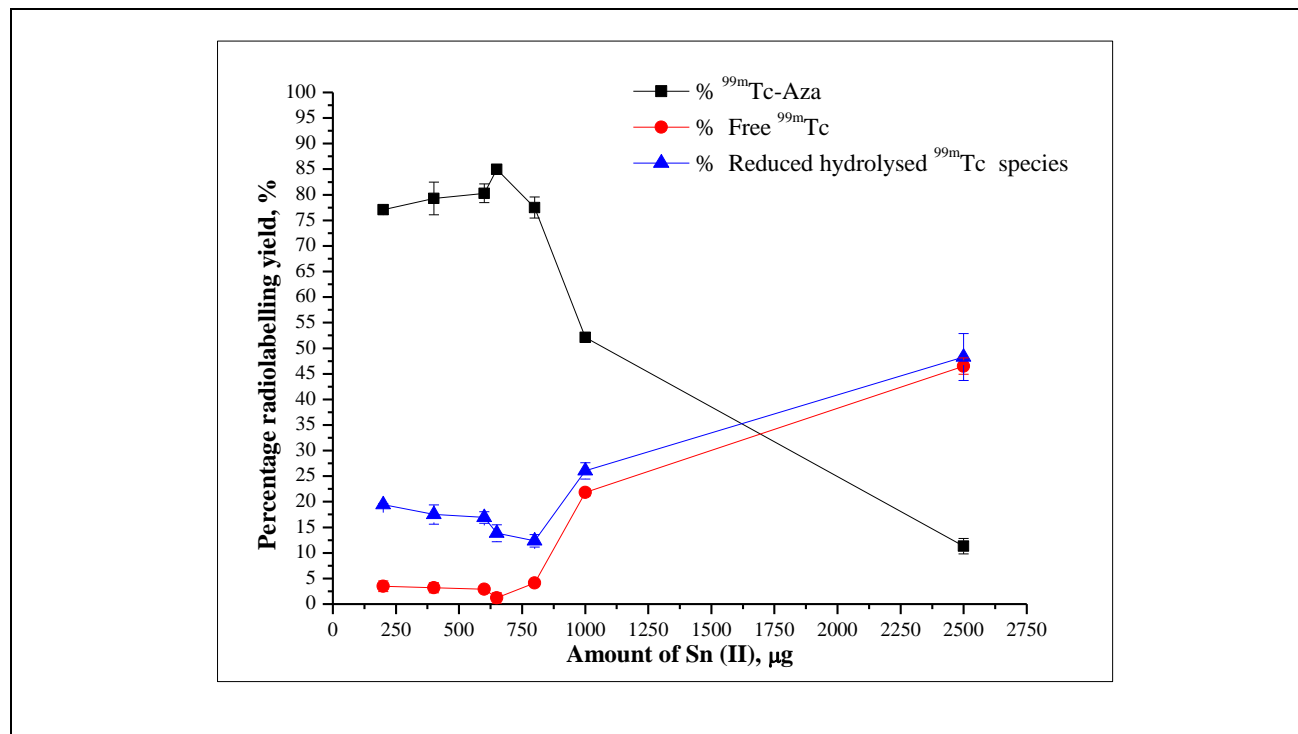
Figure 3. Effect of Sn(II) amount on the radiolabelling yield.

Reaction conditions: 650 µg of 5-Aza in solution (1mg/1ml), 100 µl $^{99m}TcO_4^-$ (~ 200 MBq) and x µg of Sn (II)at room temperature.
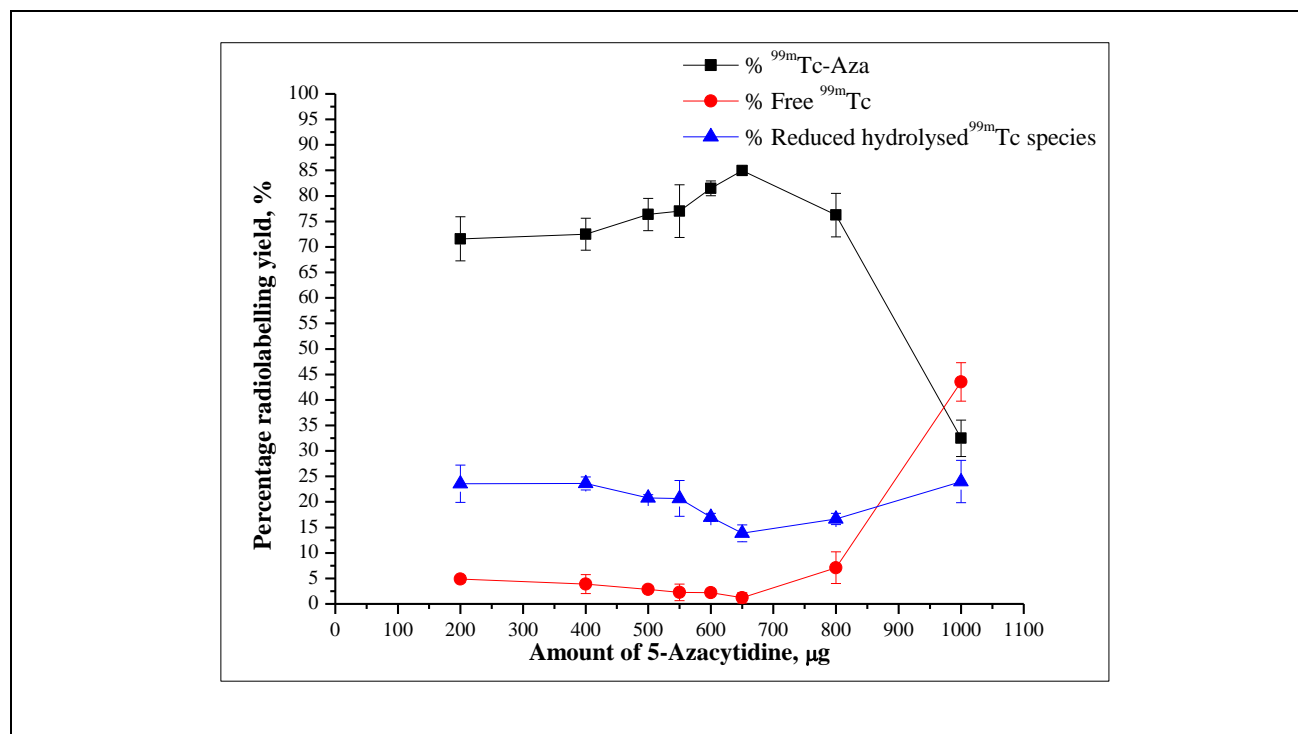

Figure 4. Effect of 5-Aza amount on the radiolabelling yield.

Reaction conditions x µg 5-Aza in solution (1mg/1ml), 100 µl $^{99m}TcO_4^-$ (~ 200 MBq) and 650µg of Sn (II) at room temperature.

RESULTS AND DISCUSSION

Radiolabeling with ^{99m}Tc or $^{186/188}$Re is dependent on complexation chemistry. ^{99m}Tc is eluted as ^{99m}TcO$_4^-$and the oxidation state of ^{99m}Tc was +7. In order to achieve complexation,^{99m}Tc must be reduced to a suitable oxidation state which ranges from +1 to +6[24]. The oxidation state of ^{99m}Tc is dependent on the reducing agent used, the reaction medium and the ligand characteristics[24]. The factors affecting the efficiency of the radiolabeling process (radiolabeling yield) such as pH of the reaction, temperature, reducing agent amount and the ligand amount (5-Aza), are studied in details.

Determination of the radiolabeling yield

The reaction yield was observed using paper chromatography and 2 system mobile phases. The first mobile phase was acetone where the ^{99m}Tc-Aza complex along with the reduced hydrolysed species remained at the origin ($R_f = 0$) and the free reduced ^{99m}Tc had R_f value of 0.7. The second system used normal saline as mobile phase where the reduced free ^{99m}Tc along with the ^{99m}Tc-Aza complex travelled with the solvent front while the reduced hydrolysed species remained at the origin. The final radiolabeling yield can be calculated by subtracting the percent of reduced hydrolysed^{99m}Tc species from paper 2 from the combined percentage of ^{99m}Tc-Aza and reduced hydrolysed^{99m}Tc species from paper 1[14].

Effect of pH and temperature of the reaction

5-Aza is considered to be unstable in aqueous solution. From previous literature, it was found that, the aqueous solution of 5-Aza is stable in pH solutions with values close to 6 when kept at ambient temperature. At high temperatures ($> 25^0$C), the rate of formation of the degradation product N-(formylamidino)-N^1-β-D-ribofuranosylurea (RGU-CHO) of 5-Aza increases rapidly with time. RGU-CHO is then irreversibly decomposes to yield 1-β-D-ribofuranosyl- 3-guanylurea (RGU) in a continuous degradation process. The degradation process took several days at 25^0C but only a few hours when 5-Aza solution was kept at 80^0C. As mentioned before, 5-Aza is stable in pH solution with values near 6. At acidic pH, the decomposition of the drug was fast and the decomposition severely accelerated in basic solution to reach 10 min in pH 9.2[25-27].Depending on the data mentioned above, all the radiolabeling reactions were done at ambient temperature (25 ± 5^0C) and the pH of all the reactions was adjusted to be 6.5. However, studying the effect of these factors on the radiolabeling efficiency seemed useless.

Effect of reducing agent amount

The radiolabeling yield showed to be dependent on Sn (II)amount, as shown in **Figure 3**. The use of low Sn (II) amounts didn't generate enough reduced ^{99m}Tc to label all 5-Aza molecules this explains the moderate radiolabeling yield when 200-600 μg of Sn (II) was used. The optimum amount of Sn (II) for the labelling process was 650 μg where the radiolabeling yield was 84.94 ± 0.89 %. Increasing the amount of Sn (II) caused a rapid decrease in the radiolabeling yield. This can be explained by the decrease in the pH of the reaction towards the acidic side, causing the decomposition of 5-Aza as stated earlier.

Effect of 5-Aza amount

During this study, it was noticed that the colloidal ^{99m}Tc species are formed in high percentages which leads to the conclusion that; the substrate amount is not high enough to form a chelate with all the reduced ^{99m}Tc. The amount of 5-Aza used in this study is higher than that used in Sn (II) to compensate for the hydrolysis of the compound in water and to increase the radiolabeling yield. The results are shown in **Figure 4**, where the use of low amounts of 5-Aza (200μg) gave a low radiolabeling yield (71.59 ±4.32%) with high percent of reduced hydrolysed^{99m}Tc species (23.55 ± 3.67%). Increasing the amount of 5-Aza lead to the decrease of the reduced hydrolysed species and the radiolabeling yield increased. A maximum radiolabeling yield of 84.94 ± 0.89 %was achieved when 650μg of 5-Aza was used. A Further increase in the amount of 5-Aza caused declining in the radiolabeling yield to reach 32.49 ± 0.35 % when 1000 μg of 5-Aza was used.

Reaction time and *in vitro* stability studies

The reaction was kept at ambient temperature (25 ± 5^0C) for 120 min and samples were withdrawn at different time intervals (5-120 min) and the radiolabeling yield was estimated as mentioned before. As shown in **Figure 5**, the radiolabeling yield is time dependent, the radiolabeling yield reached its maximum value at 20 min after the addition of the reducing agent. The radiolabeling yield started to decrease after 20 min to reach 61.92± 2.37% at 120 min. This finding indicates that the formed ^{99m}Tc-Aza complex is not stable at ambient temperature. The reaction was performed as stated above and kept at ambient temperature for 20 min, then the reaction vials were transferred to the refrigerator (15^0C) for 120 min. The stability of the complex was improved but not significantly with minimal reduced hydrolysed species formation as elucidated in **Figure 6**.
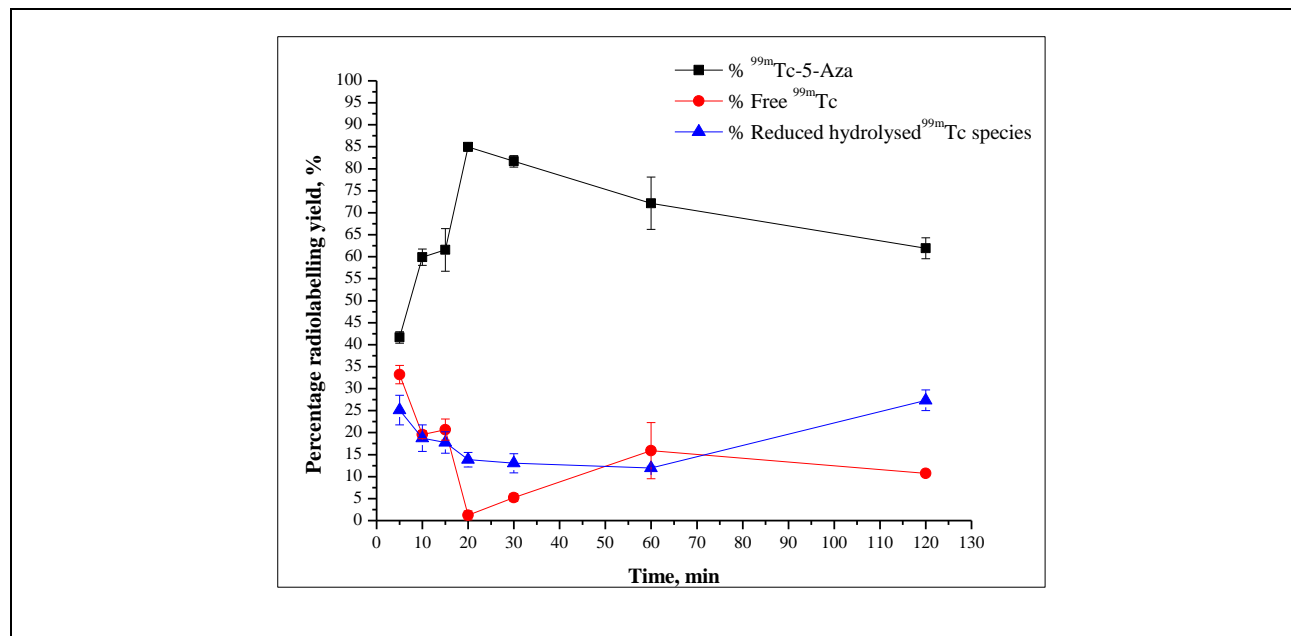
Figure 5. Effect of reaction time and on the radiolabelling yield.

Reaction conditions 650μg 5-Aza solution (1mg/1ml), 100μl $^{99m}TcO_4^-$ (~ 200 MBq) and 650μg of Sn (II)at room temperature.
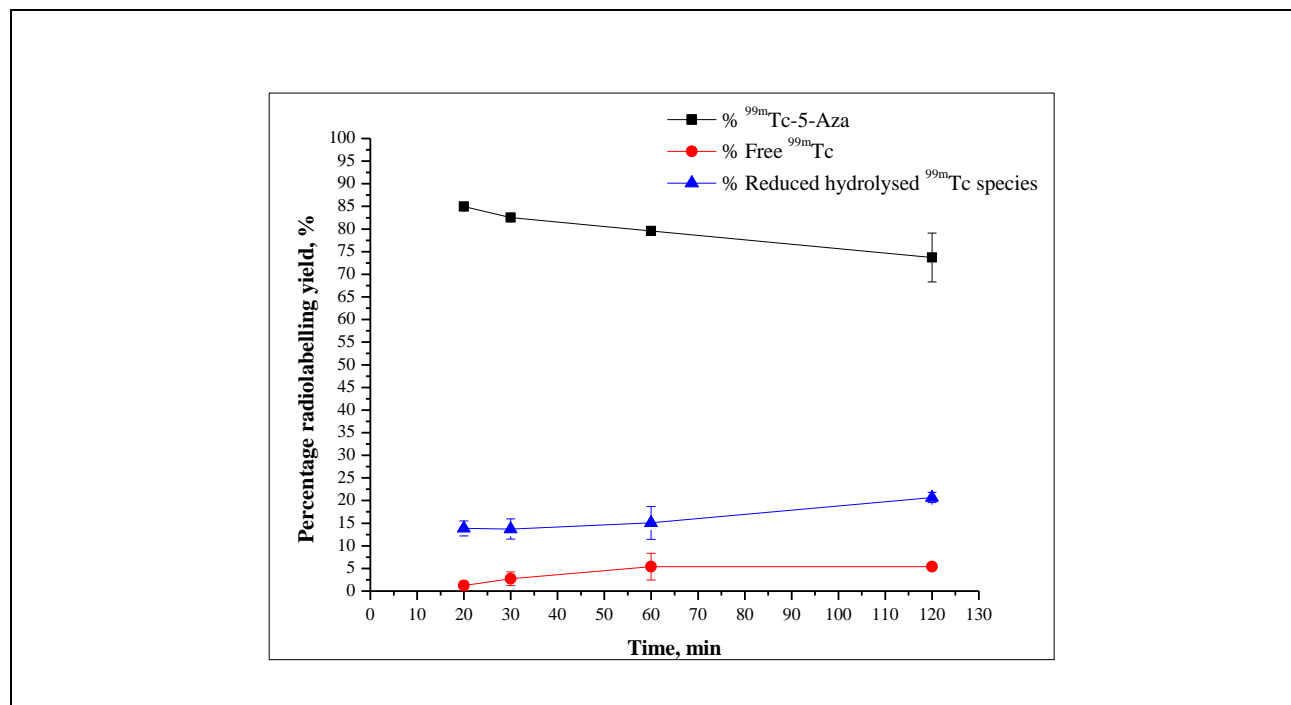

Figure 6. *In vitro* stability of ^{99m}Tc-Aza in refrigerator.

Reaction conditions 650μg 5-Aza solution (1mg/1ml), 100μl $^{99m}TcO_4^-$ (~ 200 MBq) and 650μg of Sn (II) at ambient temperature for 20 min then the reaction was kept at refrigerator for 120 min.
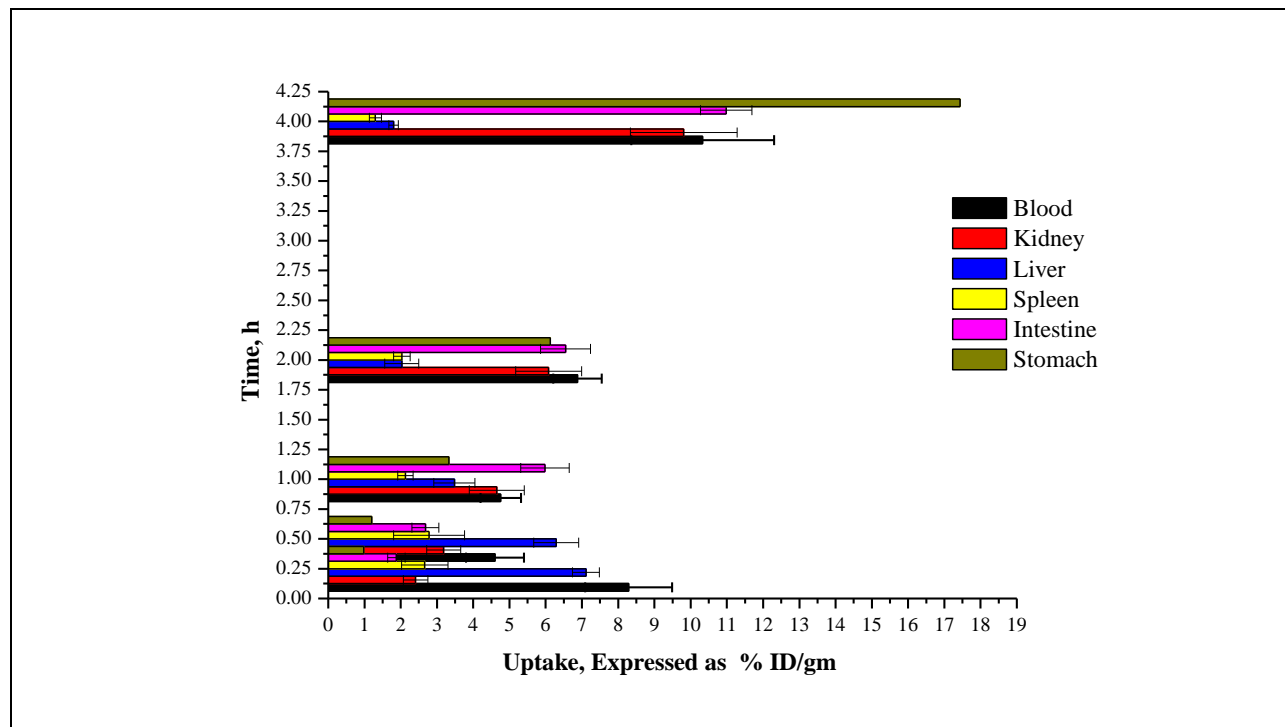
Figure 7. Uptake of ^{99m}Tc-Aza in blood, kidney, liver, spleen, intestine and stomach at different time intervals (values expressed as % ID/gm)

Biodistribution of ^{99m}Tc-Azacytidine

Biodistribution of ^{99m}Tc-Aza and the extent of its localization in the tumour site were examined by injecting the radiolabeled complex intravenously in mice bearing solid tumours. The tracking of the deposition of^{99m}Tc-Aza was assessed 0.25, 0.5, 1, 2 and 4 h after administration. Ehrlich carcinoma is the correspondent of murine mammary adenocarcinoma. It has been chosen as a tumour model for this biodistribution study due to its ease of transplantation, aggressiveness and rapid growth[28,29]. As mentioned earlier, the purpose of the radiolabeling of 5-Aza with ^{99m}Tc was to study the possibility of its future radiolabeling with $^{188/186}$Re for radiotherapeutic use. For this purpose, a good targeting must be ensured. In this case, the target/non-target (T/NT) ratio was used to express the degree of localization and targeting. A targeted agent would have high target to background ratio. The distribution of ^{99m}Tc-Aza in blood, Kidney, Liver, Spleen and intestine is shown in **Figure 7**. The distribution pattern in the blood shows initial high uptake at 0.25 h post injection (8.29 ± 1.2 % ID/gm) this uptake decreased with time (4.6 ± 0.8 %ID/gm at 0.5h post injection) which is nearly half the initial blood uptake, a finding that is consistent with the literature of peak plasma concentration of 5-aza following i.v. bolus where the plasma $t_{1/2}$ is 21 min post injection[8].5-Aza is excreted mainly through the kidney, so it was expected

to find a high accumulation of the radioactivity in the kidney. The kidney uptake was initially low and increased with time to a maximum value of 9.81 ± 1.47 % ID/gm at 4 h post injection in comparison with 2.41 ± 0.34 at 0.25 h post injection, which can be explained by the excretion of the complex mainly *via* urinary pathway mainly [8].The liver uptake was initially high (7.11 ± 0.37 % ID/gm) at 0.25 h post injection, this uptake gradually decreased with corresponding low intestinal uptake. This finding can be explained by the affinity of the complex to the liver without corresponding hepatic metabolism. On the other hand, the stomach uptake increased with time suggesting the decomposition of the complex *in-vivo* as the stomach is considered as primary target for the free ^{99m}Tc along with the intestine where it is secreted with their mucosa[14].

The initial tumour muscle uptake was relatively high (3.63 ± 0.63 % ID/gm) 0.25 h post injection when compared to the initial tumour uptake of ^{188}Re-Lanreotide (1.10 ± 0.57 % ID/gm) and ^{125}I-Cladribine (1.4 ± 0.5 %ID/gm)[17] at the same time point. The tumour uptake reached its peak 2h post injection (5.09 ± 1.01 %ID/gm) in comparison to the maximum uptake of ^{188}Re-Lanreotide (3.1 ± 0.57 %ID/gm at 1.5 h post injection)[18] and ^{125}I-Cladribine (2.8 ± 0.4% ID/gm 1 h post injection). The target (tumour muscle, T)/ non-target (contralateral normal muscle, NT) was not high
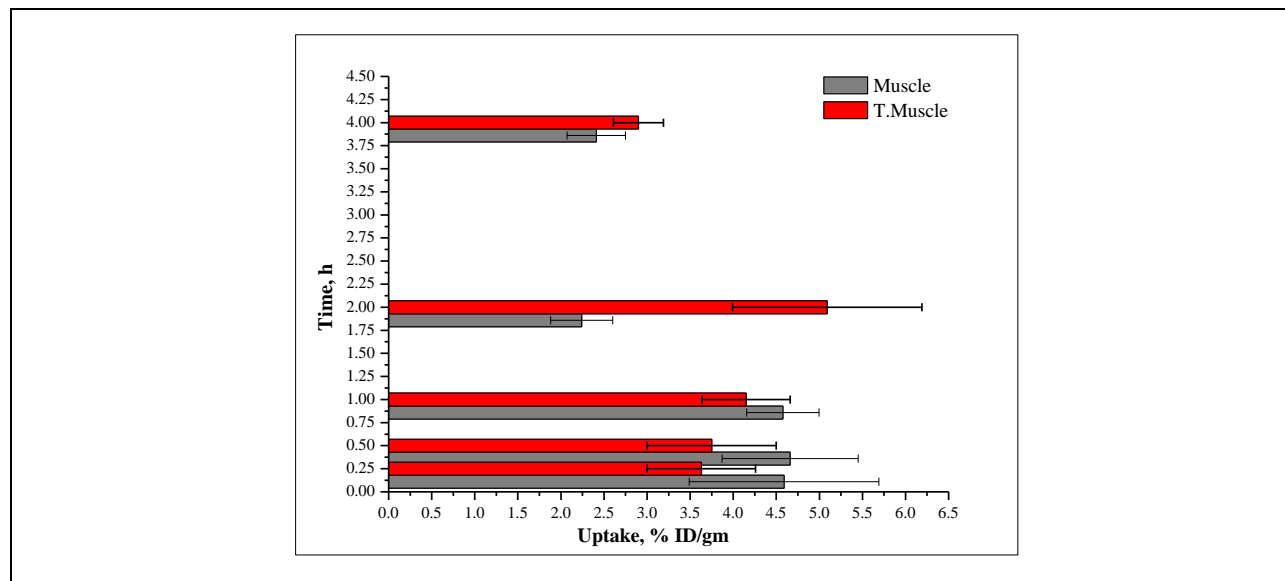
Figure 8. Uptake of ^{99m}Tc-5-Aza in tumour muscle, contralateral normal muscle.
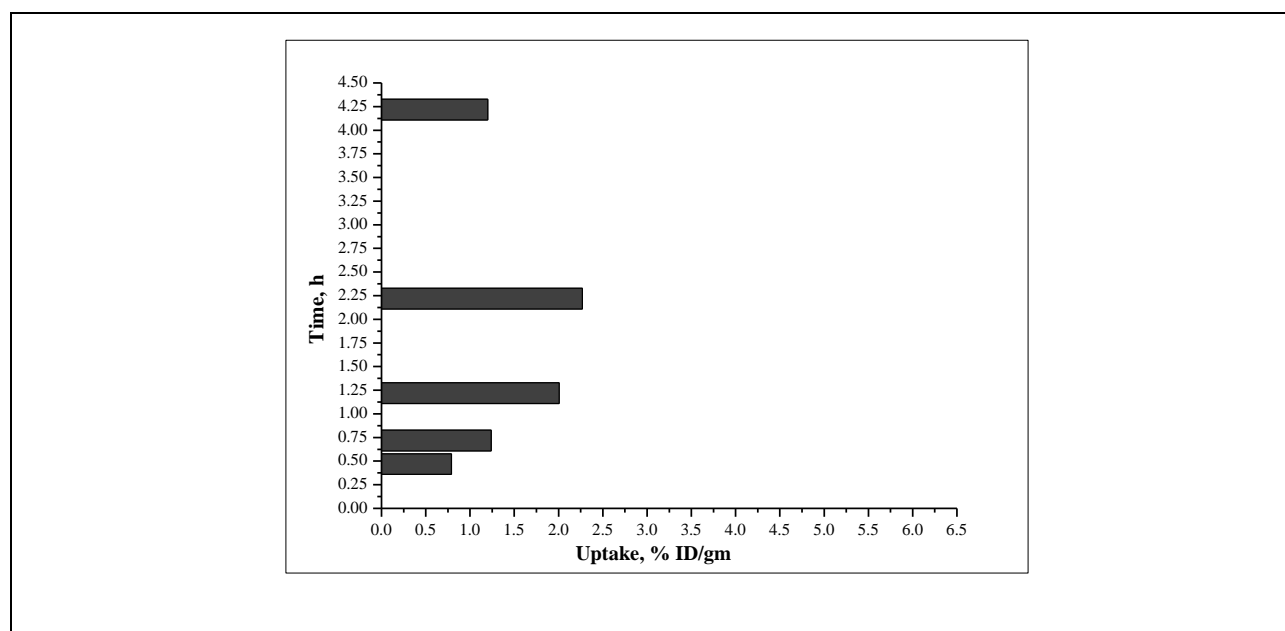

Figure 9. Target / non target(T/NT) ratio at different time intervals.

as expected due to high normal muscle uptake at times 0.25, 0.5 and 1h post injection. The normal muscle uptake was then decreased to reach 2.24 ± 0.36 %ID/gm 2h post injection and remained almost constant this led to the rise of T/NT ratio to reach 2.27 at 2 h post injection. Although the tumour muscle uptake is almost twice the uptake of the normal muscle the uptake cannot be considered high enough to consider the radiolabeled 5-Aza as targeting therapeutic agent. To ensure that further studies such as cell-viability

assay or inclusion of the radiolabelled complex in targeted pharmaceutical formulation must be conducted in future work.

CONCLUSION

5-Azacytidine was successfully radiolabeled with ^{99m}Tc in a good radiolabeling yield (84.94%). The biodistribution of the radiolabeled complex in EAC solid tumour bearing mice showed high accumulation in

the tumour muscle 2 h post i.v injection a considerably higher uptake than [188]Re-Lanreotide and ^{125}I-Cladribine. This finding supports the believe that ^{99m}Tc-Aza complex can be used for radiotherapy. However the T/NT ratio of the drug was not high enough to consider it a targeted radiotherapeutic agent due to its accumulation in normal muscles. The targeting of the drug can be improved by inclusion of the radiolabeled complex in a pharmaceutical formulation e.g. micelle or liposome to reduce its accumulation in non-target tissues.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

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