DETECTION OF SENTINEL LYMPH NODE - BASED ON SPECIFIC BINDING TO MANNOSE RECEPTORS*

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Abstract. The use of $^{99m}$Tc for developing cancer specific radiopharmaceuticals aims to translate effective products to clinical use. A class of radiopharmaceuticals, based on size accumulation is presently utilized for sentinel lymph node (SLN) detection: $^{99m}$Tc-sulfur colloid, filtered $^{99m}$Tc-sulfur colloid and microcolloids of $^{99m}$Tc-labelled albumin but none of these agents has ideal properties regarding the selective accumulation in sentinel node. Moreover, these compounds are uptaken also by distal lymph nodes. A lymphoscintigraphy agent requires high density of receptor substrate sites to achieve a specific receptor affinity required for a proper sentinel node detection. The solution could be given by mannose receptor-binding radiopharmaceuticals which can be synthesized with high specific activities compatible with typical target tissue receptor densities.

The radiolabelling of the mannosyl-cysteine-dextran macromolecules with $^{99m}$Tc (Dextran-S-Cysteine-Mannose) resulting in a high purity and stability radiolabelled conjugate, suitable for sentinel node detection with low distal (second) lymph node accumulation, as well as their in vivo biological evaluation are the aims of this study.

Different radiolabelling strategies, including novel $^{99m}$Tc cores, were evaluated in order to select and optimize the most efficient and specific one. The quality control of radiolabelled conjugates using TLC and HPLC was performed; the radiochemical purities (RCPs) of the probes were in the range 93–99 %. The biological evaluation (ex-vivo biodistribution and specific uptake) was performed on Wistar rats sacrificed at 15, 30 and 60 min post injection (pi). The biological data show a rapid and highly specific sentinel node accumulation, up to 11 % ID, and a very good sentinel node extraction in respect of the second node in the chain up to 94 % at 1h pi.

Key words: sentinel lymph node detection, dextran derivatives, mannose receptors, $^{99m}$Tc-radiopharmaceuticals.

1. INTRODUCTION

The pre-operative lymphoscintigraphy is essential and leads to the identification of the lymph node chains that may present metastatic disease [1]. The intra-operative gamma detection permits easier localization of the sentinel lymph node in a less aggressive dissection [1, 2]. It is desirable to make the intra-operative search of the same radiopharmaceutical injected for the pre-operative lymphoscintigraphic examination [3]. It is established a narrow relation between the size of a radiopharmaceutical and its properties of diffusion and delay in the lymph node: the bigger the particle, the lesser the diffusion and higher the retention in the lymph node [4]. The mannosylated macromolecules labelled with $^{99m}$Tc are investigated for targeted lymphoscintigraphy, a better uptake in the sentinel lymph node being expected due to their specific binding to mannose receptors expressed on lymph node macrophages [5].

2. MATERIALS AND METHODS

2.1. CHARACTERIZATION AND RADIOLABELLING OF DEXTRAN-DERIVED CONJUGATES CANDIDATES FOR SENTINEL LYMPH NODE DETECTION

The dextran-derived aminoterminated conjugate and the evaluated derivatives were synthetized at NCSR Demokritos, Athens. The chemical structure, probe label as well as molecular weight (MW) of starting dextran and derivatives are 22.000 MW for the derivative Dextran-S-Cysteine-Mannose (DCM-20 – with a 11.800 MW for starting dextran), two batches, and 32.000 MW for the second derivative Dextran-S-Cysteine-Mannose DCM-30 (with higher MW dextran, 20.000 MW).

2.1.2. Radiolabelling of dextran-cysteine-mannose conjugates using $^{99m}$Tc tricarbonyl core

The $[^{99m}\text{Tc}(\text{H}_2\text{O})_3(\text{CO})_3]^+$ precursor was prepared according to product leafet, by adding $[^{99m}\text{TcO}_4^-]$ generator eluent (1 mL, 0.74–1.85 GBq) to an IsoLink™ kit vial (Covidien) containing sterile lyophilized formulation, heated at 100 °C for 20 min in a water bath. After solution cooling, the pH was adjusted using HCl 1M.

The probes containing dextran-cysteine-mannose (DCM-20, DCM-30) were tested. To each probe vial, containing 400 µg dextran derived compound, 250 µL (250 MBq) of the $[^{99m}\text{Tc}(\text{H}_2\text{O})_3(\text{CO})_3]^+$ precursor (pH 8.5 – 9.0) and 250–500 µL
saline were added. Then, the probes were incubated at 70 °C for 20 min, on water bath. After cooling the radiochemical purity of the products was tested by TLC and HPLC.

2.2. QUALITY CONTROL

The radiochemical purity of $^{99m}$Tc radiolabelled dextran derivatives was determined by ascending instant thin-layer chromatography (ITLC) with silicagel-coated fibreglass sheets 20 cm length (Polygram SIL G, Macherey-Nagel, Germany) using either acetonitrile, physiological saline (0.9 % NaCl) or acidified methanol (0.1 % TFA) as the mobile phase. 5 µL samples were spotted on TLC strips and analyzed as described. Radioactivity associated with the chromatographed ITLC strips was scanned using a radiation scanner (mini Gita TLC, Raytest). The radiochemical purity of the probes was determined with GitaStar TLC software. The radioactive contaminants were identified as reduced/hydrolyzed $^{99m}$Tc, free $^{99m}$TcO$_4^-$ and $[^{99m}$Tc(H$_2$O)$_3$(CO)$_3]^+$ precursor (their Rs were determined for each solvent). The RCP of the dextran derived compounds were higher than 90 %.

Alternatively, HPLC technique (Shimadzu HPLC System) with radiation detection (Berthold) was employed for radiochemical characterization of the $^{99m}$Tc radiolabelled dextran derivatives using a Nucleosil C-18 reversed-phase column, 5µm, 250 mm × 4.6 mm (Supelco Inc.), with the following gradient:

a. Flow 1 mL/min A (0.1 %–TFA in H$_2$O), B, (0.1 %–TFA in MeOH): 0–1 min, B, 10 %, 1–28 min, B 90 %, 28–35 min, B 10 %.

b. Flow 1 mL/min A (0.1 %–TFA in H$_2$O), B, (0.1 %–TFA in CH$_3$CN): 0 min, B, 0 %; 0–25 min, B 100 %; 25–30 min, B, 100 %; 30–35 min, B, 0 %.

c. Flow 1 mL/min A (0.1 %–TFA in H$_2$O), B, (0.1 %–TFA in MeOH): 0–1 min, B, 0 %, 1–9 min, B, 70 %; 9–19 min, B, 70 %; 19–24 min, B, 95 %; 24–30 min, B, 0 %.

2.3. BIOLOGICAL EVALUATION OF THE $^{99m}$TC-RADIOLABELLED DEXTRAN DERIVED COMPOUNDS

2.3.1. Animal studies – experimental model

The experimental model consisted in young male rats from Wistar line (*Rattus norvegicus* albinos variety, rodentia, mammalia), of 200–250 g. The rats were kept in cages under ambiental temperature and humidity, receiving commercial ratio and water *ad libitum*.

The animals were anesthetized with a mixture (0.2 mL/animal) containing 0.15 mL of ketamine 10 % and 0.05 mL acepromazine (Calmivet), by injection in the peritoneal cavity using an insulin type syringe and needle. The anesthetic
concentration assures about 60 minutes of anesthesia and, if necessary, additional doses were injected.

Preliminary examinations of one rat injected with blue dye solution, 0.05 mL, in the right rear foot: at 10 min postinjection, both popliteal lymph node and deep inguinal lymph node were clearly observed being blue painted (Fig. 1).

![Fig. 1 - Visualization of popliteal lymph node after administration of blue dye.](image)

### 2.3.2. Biodistribution procedure

The rats \((n = 3)\) were subcutaneously injected in the rear right foot pad with 0.05–0.1 mL \((7–15 \text{ MBq})/\text{animal }^{99m}\text{Tc}-\text{dextran-derived conjugate. The rats were sacrificed at 15, 30, 60 and 180 min pi. At 45 min pi, the rats were laterally and medially near to the knee joint of the right rear foot, injected with methylene blue solution, particularly, } 2 \times 0.05 \text{ mL. The popliteal lymph node, the inguinal lymph node, the right rear foot pad, blood and liver samples were prelevated and placed into pre-weighted plastic tubes. All probes were weighted and counted (gamma counter Raytest). The percentage of the injected dose per g of organ (% ID/g) and the percentage of injected dose per organ (% ID) were calculated as mean values from individual measurements.}\n
### 2.3.3. In vivo uptake testing by autoradiography

The animals were injected in the foot pad, under anesthesia, with 100 µL \((10–15 \text{ MBq})\) of \(^{99m}\text{Tc}\)-radionlabelled dextran derived compounds. Groups of 2 rats were imaged 5, 10, 30 and 60 minutes thereafter. For the autoradiography, the animals were simultaneously placed under anesthesia on a storage phosphor screen MP (Perkin Elmer Life Sciences, USA) and left there in the dark for 15 to 60 s, depending on activity. In addition, an optical photography of animals was taken. The autoradiographies were developed using a Cyclone Phosphor Imager (Perkin
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Elmer Life Sciences) and analyzed using Optiquant software (Perkin Elmer Life Sciences). The position of the radioactive areas was matched by overlapping autoradiographies and photographies.

2.3.4. Lymphoscintigraphy

For the lymphoscintigraphy, 0.05–0.1 mL of the radiopharmaceutical (7–18.5 MBq) were intradermally injected into both rear footpads of the rats. The acquisitions were done in dual detector e.cam Signature Series gamma camera (SIEMENS Medical Solutions Inc., USA), using LEHR collimators, anterior detector only, matrix size 256×256, zoom 2 (1 min/frame; 60 frames) for dynamic studies and matrix size 512×512, zoom 2 for static studies. The optimal distance between collimator and animal was 10 cm.

3. RESULTS AND DISCUSSIONS

3.1. AUTORADIOGRAPHY STUDIES

After successful labelling of dextran derived compounds with $^{99m}$Tc, using the novel approaches, we explored their imaging on in vivo system. The autoradiographies and their semiquantitative analysis, presented in Figs. 2 and 3, were analyzed the results being summarized in Table 1. The regions of interest were selected, corresponding to radioactive areas, the luminescence units (DLU) per mm² being reported as well as the percentage of sum of regions. The autoradiographies show a high concentration of dextran derived compounds DCM-20, DCM-30 in sentinel (popliteal) lymph node.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Autoradiographs analysis</th>
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<tr>
<td>DCM20</td>
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<tr>
<td>SLN</td>
<td>112,566.0</td>
</tr>
<tr>
<td>2 LN</td>
<td>–</td>
</tr>
<tr>
<td>Liver</td>
<td>–</td>
</tr>
<tr>
<td>Bladder</td>
<td>–</td>
</tr>
<tr>
<td>DCM30</td>
<td></td>
</tr>
<tr>
<td>SLN</td>
<td>68,313.5</td>
</tr>
<tr>
<td>2 LN</td>
<td>–</td>
</tr>
<tr>
<td>Liver</td>
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<table>
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<tr>
<th></th>
<th>5 min DLU/mm²</th>
<th>region%</th>
<th>10 min DLU/mm²</th>
<th>region%</th>
<th>30 min DLU/mm²</th>
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<td>SLN</td>
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<td>166,465.0</td>
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<td>595,446.4</td>
<td>77.1</td>
<td>202,927.8</td>
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<td>–</td>
<td>–</td>
<td>131,360.4</td>
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<tr>
<td>Liver</td>
<td>–</td>
<td>–</td>
<td>96,359.7</td>
<td>22.3</td>
<td>325,381.2</td>
<td>6.6</td>
<td>107,051.4</td>
<td>30.2</td>
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<tr>
<td>Bladder</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>282,275.8</td>
<td>16.3</td>
<td>104,584.1</td>
<td>6.3</td>
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<tr>
<td>SLN</td>
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<td>100.0</td>
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<td>100.0</td>
<td>242,250.4</td>
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<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>126,039.9</td>
<td>9.3</td>
</tr>
<tr>
<td>Liver</td>
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<td>–</td>
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3.2. BIODISTRIBUTION STUDIES

The biodistribution studies confirm the preliminary in vivo evaluation and gave more accurate quantitative uptake data. The ID/g for prelevated organs: blood, liver, sentinel (popliteal) lymph node and second (iliac) lymph node, and the injection site were also represented for a comparative evaluation. Very good specific uptake of DCM-30 (5.8 % ID) in the popliteal lymph node was observed, corresponding to 173 % ID/g organ. DCM-30 have small uptake in the iliac lymph node (0.08 % ID); the popliteal extraction for this compound was higher than 96 %. DCM-20 was uptaken very rapidly in SLN, up to 8.43 % ID at 30 min post injection; the uptake is stable for 3 h. There is very little or no uptake in the second lymph node but, after 30 min post injection, the product accumulates in liver (9.3 % ID), bladder and kidneys (7.8 % ID). The data are presented also in Figs. 4 and Fig. 5.
Both static and dynamic acquisition were done for imaging studies of $^{99m}$Tc radiolabelled dextran derived compounds. We explored their uptake, clearance and in vivo stability from the injection in experimental model up to 3 h post injection. Some of the most significant SPECT images are presented in Figs. 6–10. The time-activity accumulation curves in SLN and in wholebody were determined for each compound.

Analyzing the images, an optimal in vivo behaviour was observed for the studied compounds: a fast accumulation in sentinel (popliteal) lymph node (Figs. 6, 9b) of 15–30 min, a low accumulation in critical organs (Figs. 7, 9a) and a very good in vivo stability, up to 3 h post injection (Fig. 8).
Fig. 6 – Dynamic SPECT images of DCM-20 in rats at 0–15 min post injection.

Fig. 7 – Dynamic SPECT images of DCM-30 in rats at 30–45 min post injection.
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Fig. 8 – Dynamic SPECT images of DCM-30 in rats at 3 h post injection.

Fig. 9 – Time-activity accumulation curves of DCM-30 in: a. whole body and b. popliteal lymph node.

Fig. 10 – Dynamic SPECT images of DCM-30 in rats at 31–45 min post injection.

The SPECT images of DCM-20 are presented in Figs. 11–16. The time-activity accumulation curve in SLN and in whole body are presented in Fig. 14. The acquisitions were made using both detectors (anterior and posterior view) and the injection site was not shielded. A high and stable uptake in SLN can be observed. Bladder, kidneys and liver are also imaged at latter stages (30 min to 1h).
Fig. 11 - Dynamic SPECT images of DCM-20 at 0–15 min pi (anterior view).

Fig. 12 - Dynamic SPECT images of DCM-20 at 15–30 min pi (anterior view).
3.4. SIZE DISTRIBUTION MEASUREMENTS

All the probes were dissolved in physiological saline (2 mL) resulting in different concentrations, as different quantities of compound were in vials (50 µg DCM-20). The size distribution measurements (Fig. 17) revealed formation of aggregates as early as 30 min after preparation and increasing the size of the particles from 7–10 nm to 230–310 nm and even to 700 nm. That was the case for all preparations: at DCM-20 it was observed a rapid switch to 314 nm and small
number of aggregates having more than 5000 nm (240 nm after sonication); these results rise the question about influence of the aggregate size on biological behaviour, that should be investigated.

Fig. 17 – Aggregate size distribution (by intensity) of DCM-20 in physiological saline solution (before and after sonication).

4. CONCLUSIONS

Very good specific uptake of DCM-30 (5.8 % ID) and DCM-20 (5.5 % ID) in sentinel lymph node as well as small uptake in the second lymph node (0.17 % and 0.08 % respectively) was observed.

The popliteal extraction of these compounds was higher than 96 %.

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