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Research Article

Bioavailability of ^{99m}Tc-Ha-paclitaxel complex [^{99m}Tc-ONCOFID-P] in mice using four different administration routes

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Summary

Paclitaxel, an anti-tumour drug, shows good results against breast and ovarian cancer. However, its therapeutic response is associated with toxic side-effects caused by the agent used to dissolve it. Recently paclitaxel was linked to the linear polysaccharide hyaluronic acid (HA), showing good solubility, stabilization, localization and a reduction of cytotoxic side-effects.

To study potential therapeutic applications, HA-paclitaxel bioconjugate (ONCOFID-P) was labelled with ^{99m}Tc by the addition of ^{99m}Tc-pertechnetate, $SnCl_2$ and sodium gluconate. The reaction mixture was incubated for 90 min at 65°C and purified by size exclusion chromatography. The obtained ^{99m}Tc-ONCOFID-P had 100% radiochemical purity and was stable in a phosphate buffer dilution 1:100 for 6 h at 37°C. ^{99m}Tc-ONCOFID-P bioavailability studies were carried out in healthy mice using four different administration pathways. The analysis showed that after intravenous administration more than 80% of the injected radiopharmaceutical was found in liver and spleen. Intraperitoneal, intravesical and oral administrations showed that all the ^{99m}Tc-ONCOFID-P remained at the administration site.

These results demonstrate that ONCOFID-P administered intravenously could be used for liver metastasis therapy due to its high physiological and receptor-specific liver uptake, while intravesical, intraperitoneal and oral administration of ONCOFID-P could be used for local treatment of superficial cancers. Copyright © 2006 John Wiley & Sons, Ltd.

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Introduction

The main objective in cancer therapy is to find a selective cytotoxic agent to target tumour cells with minimal side-effects. One of the novel chemotherapeutic drugs is paclitaxel, an anti-tumour agent which disrupts the dynamic equilibrium within the microtubule cell system and blocks the late G2 and M phase of the cell cycle. Since the microtubules are also involved in the cytodynamic processes of cell dissemination and implantation, paclitaxel also acts as an anti-metastatic drug.[1] Therefore, it has been successfully used against breast and ovarian cancer. However, the therapeutic response with the commercial formulation is always associated with toxic side-effects because it contains Cremophor EL, a castor oil/ethanol solution, essential for the paclitaxel solubility but also responsible for most of the side-effects and adverse drug reactions.[2,3]

Recently low molecular weight anticancer drugs have been linked to polymers such as hyaluronic acid[4–6] (HA), which provide advantages in drug pharmacokinetics[7] and suppresses their undesirable cytotoxic side-effects. HA is a linear polysaccharide formed by alternating D-glucoronic acid (GlcUA) and N-acetyl-D-Glucosamine (GlcNAc) units. It is one of several glycosaminoglycan components of the extracellular matrix (ECM), the sinovial fluid of joints and of the adjacent cartilage. HA acts as a signalling molecule in cell motility, inflammation and wound healing. It has also been demonstrated that the HA oligomers are specifically endocyted by chondrocytes via cell surface CD44/Hyaluronan receptors which are over-expressed in most human cancers.[8–12]

Some HA oligomers conjugated to drugs as Mitomycin C,[13] Epirubicin[14] and Butyric acid[7,15] showed advantages in drug solubility, stabilization, localization, and controlled release[16,17] and at the same time markedly enhanced the selectivity for cancerous cells.[18,19]

HA oligomer-paclitaxel bioconjugate was recently produced, showing cell-specific binding and a specific uptake in cell cultures.[20] *In vitro* evaluation of the bioconjugate showed a direct correlation between uptake and cytotoxicity. In addition, Yin *et al.*[21] demonstrated that the HA-paclitaxel complex is more effective in inhibiting metastasis, adhesion and growth of tumour cells *in vivo*, and has fewer side-effects than in the case of either using paclitaxel itself or the HA oligomer.

To assess potential therapeutic applications of the HA-paclitaxel bioconjugate (ONCOFID-P), it is necessary to determine its bioavailability. The

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gamma-emitting radioisotope ^{99m}Tc is widely used in pre-clinical studies to determine the pharmacokinetic properties of the new drugs in animals by measuring the concentration of a radiolabelled complex in each organ. In addition for small animals it is possible to obtain *in vivo* images of the drug distribution using high-spatial-resolution position-sensitive cameras such as Yttrium-Aluminium-Perovskite (YAP) type scintillation cameras.[22]

The aim of this study was to label ONCOFID-P with ^{99m}Tc and to evaluate its biodistribution after intravenous, intraperitoneal, intravesical or oral administration in healthy mice. Both *ex vivo* gamma-ray activity in selected organs and *in vivo* gamma ray image analysis have been conducted.

Experimental

The paclitaxel-hyaluronic acid bioconjugate (ONCOFID-P) has been synthesized and characterized by Fidia Farmaceutici spa (Abano Terme, Italy) as described by Rosato *et al.*[23] Briefly HA (Mw $\approx$ 200 kDa) was used to produce the tetrabutylammonium-hyaluronic salt (HA-TBA) that reacted with the paclitaxel ester formed by activation with 4-bromo butyric acid. The ONCOFID-P obtained by this method presented a substitution of 20% w/w. The chemical structure is reported in Figure 1.

All the other reagents were analytical grade and were used without further purification. ^{99m}Tc-pertechnetate was obtained from a DRYGEN ^{99}Mo/^{99m}Tc generator (Amersham-Health).

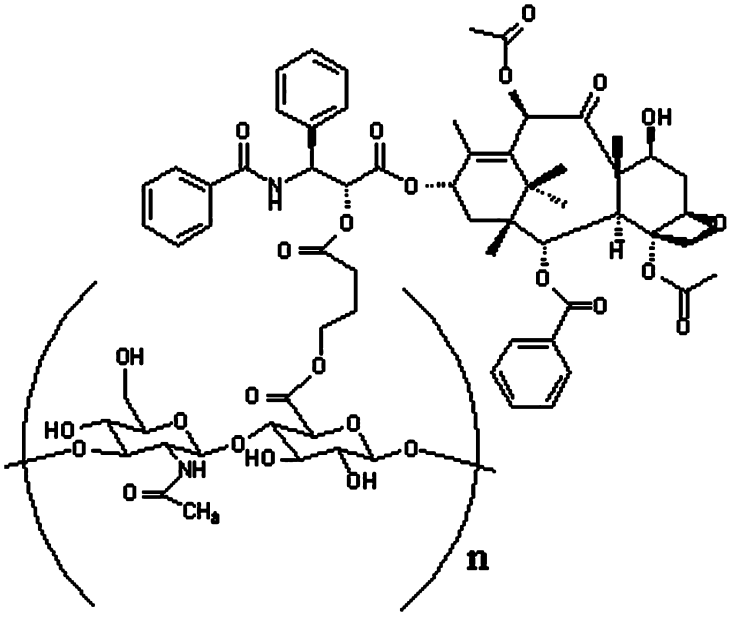

Figure 1. Molecular structure of ONCOFID-P

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Animal studies were performed according to the official Italian rules and regulation for safe and adequate laboratory handling.

Direct labelling of ONCOFID-P bioconjugate

ONCOFID-P bioconjugate was radiolabelled by the addition of 100 µl (100 MBq) of ^{99m}Tc-pertechnetate solution to a vial containing $SnCl_2$ and sodium gluconate followed by 50 µl (2.38 mg/ml) ONCOFID-P solution. The pH was adjusted to 4 and then the preparation was gently mixed and incubated at various temperatures and time intervals. The pH of the reaction mixture was maintained to 4 since it was proven experimentally that this value was low enough to allow the TcO_4^- reduction using $SnCl_2$ without causing the split of HA and paclitaxel contained on ONCOFID-P complex.

In order to optimize the formulation the following parameters were examined: the amount of sodium gluconate (6, 8, 10, 12, 14 and 16 nmol); the amount of stannous chloride (80, 100, 120, 134, and 158 nmol); the reaction temperature (25, 35, 50, 65, 80 and 90°C); and the incubation time (30, 60, 90, and 120 min). Only one parameter was altered at a time.

Quality control

Radiochemical purity analyses were performed using size exclusion chromatography and instant thin layer chromatography (ITLC).

Size exclusion chromatography was carried out using a Hi-Trap desalting column (void volume 1.5 ml, Supelco; Sigma-Aldrich) with a 1000–5000 Da cut-off. The ^{99m}Tc-ONCOFID-P there was quickly eluted with water from the column while the Sephadex G25 resin retained ^{99m}Tc-pertechnetate and other low-weight species.

ITLC on silica gel impregnated glass fibre sheets (Pall Italia s.r.l., Milan, Italy) was carried out by running a sample of 1 µl on a 10 cm strips using acetone, saline solution, and a mixture 50% of 0.1 M sodium citrate pH 5 and 50% acetonitrile (ACN) as mobile phases. Distribution of radioactivity on the strip was determined cutting it into 1 cm pieces and each one was counted in a NaI-scintillation counter. R_f of ^{99m}Tc-ONCOFID-P is 0.0 in acetone and in saline solution and 1.0 in sodium citrate/ACN. The R_f of the labelled ^{99m}Tc-gluconate is 0.0 in acetone and 1.0 in both saline and sodium citrate/ACN, while free pertechnetate migrates with the front in the three solvents ($R_f = 1.0$). Reduced hydrolysed ^{99m}Tc stays at the seeding point with the three solvents ($R_f = 0.0$).

Dilution stability

To determine the stability of ^{99m}Tc-ONCOFID-P complex after dilution, 100 µl of purified complex was diluted to a ratio 1:100 in phosphate buffer

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0.1 M (pH 7.4) and incubated for 6 h at 37°C. The radiochemical purity of the complex was analysed every hour by ITLC.

Biodistribution studies

The biodistribution of ^{99m}Tc-ONCOFID-P was done in four groups of five normal female C57BL/6 black mice (Charles River Italia, Calco, Como, Italy). All of them were given 200 µl (~13 MBq) of the labelled complex, but using four different routes of administration. To the first group the administration was done intravenously, to the second intraperitoneally, to the third intravesically and the last group of mice received an oral administration. The distribution of the ^{99m}Tc-ONCOFID-P in the mice was observed using a gamma-ray scintillation camera based on a highly segmented scintillator pillars from YAP. The anesthetized mice have been placed in the field of view of the YAP camera. The dynamics of the biodistribution of ^{99m}Tc-ONCOFID-P in the organs has been studied for the first 2 h after the administration analysing the gamma-ray images obtained at time interval of 5 min. The animals were sacrificed after 2 h in a CO_2 chamber and selected organs (thyroid, blood, heart, lung, stomach, liver, spleen, kidney, intestine and bladder) were removed and weighed. The total gamma-ray activity of these organs was measured in a well-shaped scintillation gamma-ray counter. A fixed quantity of ^{99m}Tc-ONCOFID-P has been measured in the gamma-ray counter in order to deduce the total amount of the activity administrated to the mice. The radioactivity of the tissue samples was expressed as percentage of the injected activity per gram of tissue (% IA/g). The observations from the acquired images for the accumulated organ activity were correlated with the percentage of IA/g obtained by the *ex vivo* experiment.

Results and discussion

Radiolabelling yield

The reaction temperature had the most significant effect on the radiolabelling yield. This result can be explained by the fact that the ONCOFID-P solution is quite viscous at room temperature. The complex becomes more fluid with the temperature increase, thus leading to a higher possibility of interaction with the ^{99m}Tc present in the reaction mixture (Figure 2). Since ONCOFID-P can be degraded at temperatures higher than 80°C, it was decided to incubate at 65°C. The analysis of the influence of incubation time on the radiolabelling yield was studied and the results showed an increment of the yield proportional to the incubation time with a maximum value after 90 min (Figure 3).

The amount of reducing agent ($SnCl_2$) produced a minimal effect on the radiolabelling yield probably because the reduced ^{99m}Tc (rapidly generated by

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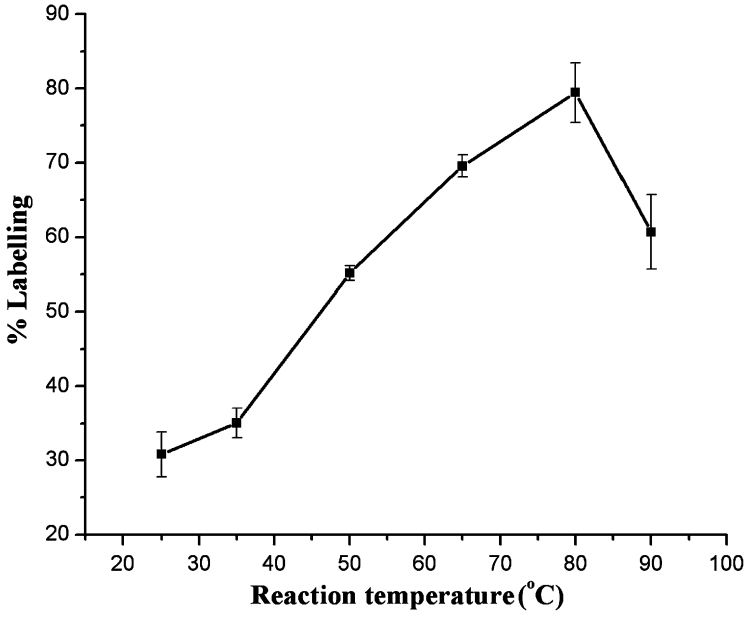

Figure 2. Percentage yield of [^{99m}Tc-ONCOFID-P] analysed by size exclusion chromatography using 100 µl (100 MBq) of ^{99m}Tc-pertechnetate solution, 1.4 µl of a 0.1 M $SnCl_2$ solution (in 0.1 M HCl), 0.8 µl of a 10^{-2} M sodium gluconate solution and 50 µl of 2.38 mg/ml ONCOFID-P solution, incubated at pH 4 for 90 min at different temperatures

the $SnCl_2$ added in excess) was quickly stabilized by the HA polymer until the reaction was complete. Minimal effect on the radiolabelling yield was also caused by the addition of sodium gluconate. This can be explained by the fact that this agent was used as an exchange ligand to avoid the formation of reduced hydrolized ^{99m}Tc, since the HA acts as an exchange ligand itself the presence of sodium gluconate was not critical.

The highest radiolabelling efficiency of ^{99m}Tc-ONCOFID-P assessed by size exclusion chromatography and ITLC was 70% and it was obtained when 100 µl (100 MBq) of ^{99m}Tc-pertechnetate were reduced with 1.4 µl of a 0.1 M $SnCl_2$ solution (in 0.1 M HCl) in the presence of 0.8 µl of a 10^{-2} M sodium gluconate solution and was added to 50 µl of 2.38 mg/ml ONCOFID-P solution. The pH was adjusted to 4.0. The reaction mixture was gently mixed and incubated at 65°C for 90 min.

It is difficult to determine the exact structure of the labelled complex using a direct labelling method. However, knowing the functional groups presenting in the labelled molecule a theoretical model can be proposed. It is widely known that the metal centre can be coordinated by the hydroxyl present in the carbohydrate skeleton as in gluconate, generating complexes with low stability

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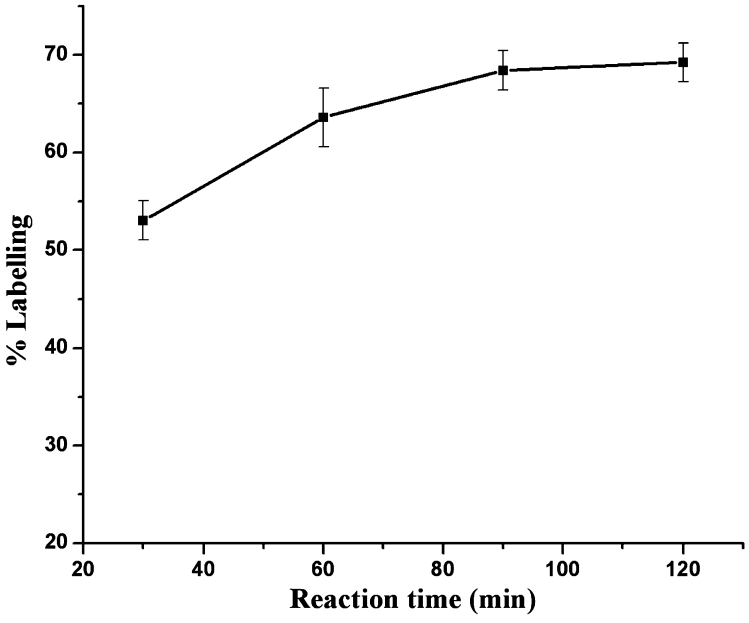

Figure 3. Percentage yield of [^{99m}Tc-ONCOFID-P] analysed by size exclusion chromatography using 100 µl (100 MBq) of ^{99m}Tc-pertechnetate solution, 1.4 µl of a 0.1 M $SnCl_2$ solution (in 0.1 M HCl), 0.8 µl of a 10^{-2} M sodium gluconate solution and 50 µl of 2.38 mg/ml ONCOFID-P solution, incubated at pH 4 and 65°C for different time intervals

and which have been used as exchange ligands in the synthesis of radiopharmaceuticals labelled with ^{188}Re and ^{99m}Tc.[24,25] However, some sugar derivatives such as glucarate labelled with ^{99m}Tc showed high stability.[26] This stability can be explained by the coordination of the metal core with both the hydroxyl and the carboxyl moieties present in the aldaric sugar derivatives. $TcO(O–COO)_2$ is the type of coordination proposed for the complex ^{99m}Tc(V)-hyaluronic acid.[27] The structural characterization of the ^{99m}Tc-oligomers of D-glucuronic acid and of N-acetyl-D-glucosamine is currently underway to confirm this hypothesis.

Purification of the ^{99m}Tc-ONCOFID-P complex and dilution stability

Radiochemical purification of the ^{99m}Tc-ONCOFID-P bioconjugate was performed before stability tests and biological studies. It was done by size exclusion chromatography using a Hi-Trap desalting column. This column perfectly separates all the possible radioactive species, and therefore its elution profile gives accurate quantification and purification of the product at the same time. ^{99m}Tc-ONCOFID-P was rapidly eluted with water from the column in the first 2 ml fraction. ^{99m}Tc-pertechnetate was eluted with the

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4–8 ml fractions, while the Sephadex G25 resin retained the reduced hydrolysed ^{99m}Tc. When a pure ^{99m}Tc-gluconate solution was passed through the Hi-Trap desalting column a low percentage of the radioactivity eluted with the 4–6 ml water fractions and the rest part remained in the column. The fact that radioactivity remained in the column matrix after elution could be explained by the instability of the complex which caused the ^{99m}Tc transchelation from the gluconate to the column dextran matrix.

The total elution of ^{99m}Tc-ONCOFID-P from the dextran column demonstrated its higher labelling stability since it avoids transchelation with the dextran matrix. The radiochemical purity of the purified ^{99m}Tc-ONCOFID-P diluted to a ratio 1:100 with phosphate buffer pH 7 was $>95\%$ after 2 h incubation at 37°C, and $>75\%$ after 6 h. At these conditions the pertechnetate is the most important decomposition product formed.

Biodistribution studies

Biodistribution studies of the purified ^{99m}Tc-ONCOFID-P bioconjugate were done in normal female C57BL/6 black mice since this strain is appropriate to implant different kinds of tumours necessary for the therapeutic studies.

Scintigraphic images obtained with the YAP camera from the group of animals injected intravenously with ^{99m}Tc-ONCOFID-P showed rapid and high liver and spleen uptake. After 25 min, 76.54 ± 1.21 and 6.46 ± 0.91 of the injected activity was accumulated in the liver and the spleen, respectively, and remained at least for 2 h, when mice were sacrificed (Figure 4). This result was explained on one hand by the lipophilic feature of the complex administrated, that instigates its fast clearance from the blood by the polygonal cells of the liver, and on the other hand by the selective uptake of hyaluronic acid via receptors present in the liver and the spleen endothelial cells.

Similar biodistributions results were found after intravenous administration of hyaluronic acid esterified with butyric acid and labelled with technetium-^{99m} (^{99m}Tc-HA-But).[24] Probably this is because the conjugation of butyric acid or paclitaxel leads to minor changes in the charges, in the conformation and the hydrophilicity, without causing significant changes in the biodistribution and the physiological interactions of a high-molecular weight polymer such as HA.

The second group of mice had an intraperitoneal administration of ^{99m}Tc-ONCOFID-P. It showed that a high percentage of the radiolabelled complex remained in the peritoneum (Figure 5). That is the reason why there was high uptake in the stomach, in the spleen and in the kidneys (see Table 1). Because of the high degree of viscosity and the large size of ^{99m}Tc-ONCOFID-P molecules only a small percentage of the injected complex was absorbed into the bloodstream.

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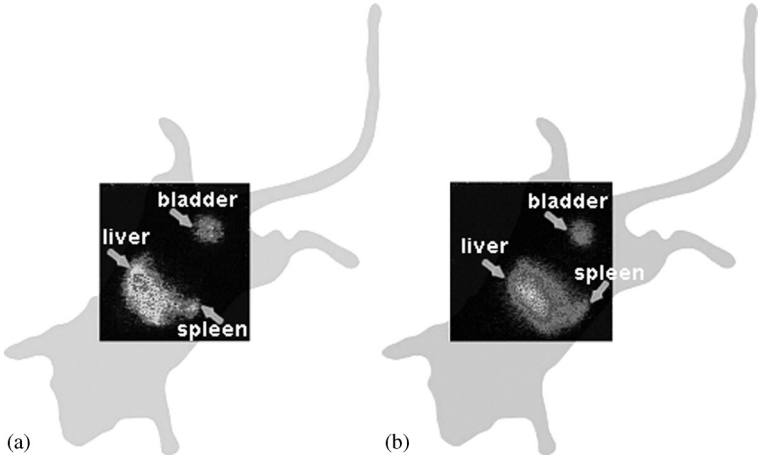

Figure 4. Biodistribution of [^{99m}Tc-ONCOFID-P] after intravenous administration: (a) image from 25 to 30 min, and (b) image from 115 to 120 min

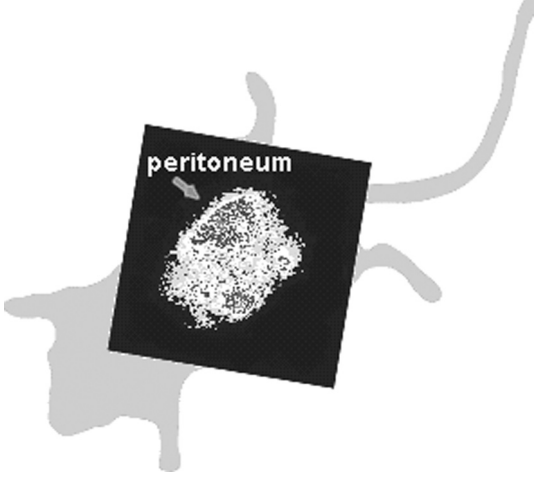

Figure 5. Biodistribution of [^{99m}Tc-ONCOFID-P] after intraperitoneal administration image from 115 to120 min

The experiments with the intravesical and the oral administration showed that more than 97% of the ^{99m}Tc-ONCOFID-P complex remained in the administration site for at least 2 h. The data for the measured total activity of the organs correlated with the obtained gamma-ray images (Figure 6).

Bioavailability is used to describe the fraction of an administered dose of medication that reaches the systemic circulation to be transported to the target site. In the case of ^{99m}Tc-ONCOFID-P the bioavailability was 100% immediately after intravenous administration as was expected. When this complex was administered by non-intravenous routes, biodistribution studies

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Table 1. Biodistribution of ^{99m}Tc-ONCOFID-P in normal female C57 black mice after different administration paths expressed as % injected activity per gram of tissue (%IA/g) ($n = 3$)

	Administration path (Mean ± SD)			
Tissue	Intravenous	Intraperitoneal	Intravesical	Oral
Thymus	0.59 ± 0.53	0.55 ± 0.49	0.68 ± 0.86	0.14 ± 0.09
Blood	1.27 ± 0.51	0.81 ± 0.41	0.08 ± 0.02	0.03 ± 0.03
Heard	1.19 ± 1.37	2.28 ± 2.30	0.53 ± 0.56	0.16 ± 0.31
Lung	1.57 ± 1.16	3.33 ± 2.39	0.50 ± 0.36	0.49 ± 0.37
Stomach	2.39 ± 1.17	17.41 ± 10.07	0.14 ± 0.17	256.28 ± 66.34
Liver	42.96 ± 1.34	6.85 ± 0.86	0.29 ± 0.14	0.20 ± 0.22
Spleen	40.67 ± 1.19	39.74 ± 15.04	1.96 ± 1.20	0.20 ± 0.08
Kidneys	1.88 ± 0.03	14.37 ± 10.71	1.69 ± 2.21	0.07 ± 0.04
Intestines	0.69 ± 0.36	6.58 ± 3.91	0.13 ± 0.03	2.74 ± 0.20
Bladder + urine	4.87 ± 0.64	1.21 ± 1.05	95.03 ± 5.51	0.10 ± 0.05

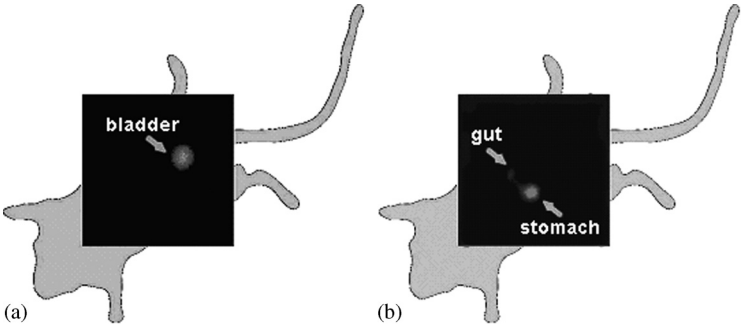

Figure 6. Biodistribution of [^{99m}Tc-ONCOFID-P] image from 115 to 120 min after: (a) intravesical administration and (b) oral administration

showed that its bioavailability decreased to less than 2% and hence it cannot be used as a systemic agent. Since the biodistribution pathways of both complexes ^{99m}Tc-ONCOFID-P and ONCOFID-P are expected to be the same because of the high molecular weight, it can be concluded that ONCOFID-P administered intravenously could be used for liver metastasis therapy due to its properties of rapid and high liver uptake. The intravesical as well as the oral administration of ONCOFID-P could be used for local treatment of bladder or stomach superficial cancers. Finally, the intraperitoneal route could find an appropriate application against ovarian cancer or other tumours spreading within the peritoneal cavity.

This study represents a first step towards the development of ONCOFID-P labelled with the beta- and gamma-ray emitting nuclide ^{188}Re (which has coordination chemistry characteristics similar to the ones of technetium) to create a new combined cancer therapeutic agent. Currently the best results in

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cancer treatment are obtained using the combined-modality therapy using an anti-tumour agent radiolabelled with a beta emitting radionuclide.[28,29]

It has been demonstrated that beta-irradiation produces not only cellular death by DNA damage like in the case of the gamma- or X-ray irradiation. It also inhibits (*in vitro*) cancer cell growth by activation of apoptosis pathways in single cells at several levels, including the triggering of ligand/receptor pathways, mitochondrial activation and caspase activation.[30] Therefore, tumour responses applying the combined therapy are higher than the chemotherapy or radiotherapy with an external gamma-ray source.

Our intention when labelling ONCOFID-P with ^{188}Re, is not to kill the cell by direct irradiation, but to induce cytotoxicity and to initiate the signalling apoptosis pathways. These effects could be added to the paclitaxel and HA combined effects on metastasis inhibition and growth tumour reduction to obtain a more effective anticancer drug.

Conclusion

^{99m}Tc-ONCOFID-P was prepared by a direct method with a radiochemical purity of 100%. The bioavailability studies conducted in this work showed that the ONCOFID-P administered intravenously could be used for liver metastasis therapy due to its high physiological and receptor-specific liver uptake. The intravesical and oral administration routes of ONCOFID-P could be used for local treatment of superficial cancers at these target organs, while the intraperitoneal route might be useful against locally growing cancers.

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