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Uptake of radiolabelled modified fragment of human α-fetoprotein by experimental mammary adenocarcinoma: *in vitro* and *in vivo* studies

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Abstract

BACKGROUND: The aim of the study was to examine *in vitro* and *in vivo* binding of radiolabelled analogues of P149 peptide by experimental mammary adenocarcinoma with the intention of potential application for diagnosis and internal radiotherapy of tumours.
MATERIAL AND METHODS: The 36-amino acid peptide (P149-QY) of 90% homology to 447–480 peptide fragment of hAFP was synthesised and radiolabelled with iodine-125. The biodistribution of P149-Q[^{125}I]-Y was studied in experimental mammary tumours. For *in vitro* experiments, extract from mouse mammary tumours were prepared and incubated with radioiodinated P149-QY peptide in the presence of a cross-linking reagent.
RESULTS: The gel electrophoresis analysis (SDS-PAGE) showed that radioiodinated P149-QY peptide formed a complex with adenocarcinoma proteins of about 30 kDa. The biodistribution of P149-Q[^{125}I]-Y studied in experimental mammary tumours revealed a higher pharmacokinetic rate in comparison with the whole radioiodinated AFP molecule. A moderate uptake of P149-Q[^{125}I]-Y in the tumour tissue was observed (3.2% ID/g at 30-min *p.i.v*). However, a faster radioactivity clearance from blood and normal tissues resulted in an increase in the tumour/muscle (T/M) ratio, i.e. from 2.3 to 3.4 after 30 mins and 24 h *p.i.v*, respectively.
CONCLUSIONS: The present study shows that radioiodinated P149-QY peptide reveals some positive features as the AFP receptor radioligand, however, some additional structural modifications of the initial peptide molecule are necessary for full retention of the ligand-receptor interaction of its radiolabelled forms.
Key words: α-fetoprotein, α-fetoprotein receptor, P149-QY peptide, radiolabelling, mammary adenocarcinoma

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Introduction

It has been postulated that the growth of some tumour cells occurs, among others, through the interaction of α-fetoprotein (AFP) with cell surface proteins. In further studies, a specific receptor for AFP (rAFP) was shown to be present on human breast cancer cells [1–7]. Expression of rAFP on human unproliferating cells was very low. The amount of rAFP molecules on lymphoma cells was about 10 times higher in comparison with normal proliferating T lymphocytes [8]. The AFP binding proteins were detectable in both soluble and membrane bound forms, revealing different molecular masses [9–11]. Therefore, attempts have been made to utilize the radiolabelled AFP molecule for *in vivo* detection of neoplastic foci [12–14]. It has been observed *in vivo* that mouse mammary carcinoma preferentially incorporated radiolabelled AFP in comparison with the normal tissue [12]. Additionally, a significantly higher accumulation of labelled [^{131}I]-AFP in mouse mammary adenocarcinoma than in rat mammary adenoma has also been noticed [14]. Like other radiolabelled proteins and ma-

cromolecules, [^{131}I]-AFP revealed high retention in blood and slow pharmacokinetics, that are the main drawbacks of its application as an *in vivo* imaging agent.

AFP, like some proteins, may serve as precursor molecules, which contain multiple modular sequences, or cassette segments, generated by proteolytic digestion to produce smaller biologically active peptides. Pro-opiomelanocortin, among others, is a classical example of such proteins, which after cleavage at various arginine-lysine sites may be a source for biologically active hormones such as γ- α- and β-melanocyte stimulating hormones, β-endorphin, β-lipotrophic hormone and ACTH [15]. Epidermal growth factor-like (EGF) proteins can also be the source of a broad spectrum of proteins associated with cell adhesion, neural development, blood coagulation, fibrinolysis and others [15–17].

Recently, a 34-amino-acid fragment 447–480 of human AFP (P149) located on the third domain was derived, and it was shown that the peptide retained the anti-estrogenic activity [18, 19]. The peptide was identified as AFP estrogen-binding pocket, which might bind to the AFP cell surface receptor [18]. It has also been shown that P149 peptide possesses an anti-breast cancer activity and growth inhibition activity in non-tumour mouse uterine assay [18–23], and it probably plays a crucial role in binding AFP molecules to rAFP [18].

In the present study, *in vitro* and *in vivo* binding of radiolabelled analogue of P149 peptide by experimental mammary adenocarcinoma was investigated with the intention of its potential application for diagnosis and internal radiotherapy of tumours.

Material and methods

P149-QY synthesis

The 36-amino acid peptide (named P149-QY; Molecular weight: 3721 Da) of the following sequence: EEDKLLACGEGAA--DIIIGHLCIRHEMTPVNPGVG-QY-COOH was prepared by standard solid phase techniques on a benzhydrylamine resin, using Boc-protected amino acids. The peptides were cleaved from the resin and deprotected by treatment with hydrofluoric acid. The crude peptides were purified on the Vydac C_{18} reverse phase HPLC column. The purity of the peptides was estimated to be over 95%. The structure was confirmed by FAB mass spectrometry.

Radioiodination of P149-QY peptide

^{125}I-labelled P149-QY peptide was prepared by the Iodo-gen method. 0.5 mg of P149-QY peptide was dissolved in 20 μl of 0.2 M aqueous Na_2CO_3. 50 μl of 0.1M phosphate buffer of pH 6.9 and 10 μl of Na^{125}I (50 MBq) solution were added. Radioiodination reaction was started by transferring the mixture into a conical vial coated with 50 μg of 1,3,4,6-tetrachloro-3α-6α-diphenyl glycoluril (Iodo-gen, *Pierce Chemical Co.*). The iodination mixture was gently vortexed for 30min at room temperature. Then 100 μl of 1% acetic acid was added, and the mixture was transferred to an SPE C_{18} column (100 mg, LiChrolut, *Merck*), previously equilibrated with 0.1 M phosphate buffer of pH 6.0. The extraction column was washed with two 0.5 ml portions of phosphate buffer (pH 6.0), and finally with 0.5 ml of redistilled water. The radiolabelled peptide was extracted from the column by three portions (3 $\times$ 0.3 ml) of 40% ethanol in 0.1 M phosphate buffer of pH 8.0.

Radiochemical analysis

The radiochemical purities of the preparations were determined by RP-HPLC and paper electrophoresis methods. HPLC conditions were as follow: Luna C_{18} 5 mm 150 $\times$ 4.6 column; mobile phase: (A) 50 mM phosphate buffer pH 10.0, (B) 100% Acetonitrile; gradient: 20% B to 100% B in 15min, 100% B for 10 min; flow rate = 0.5 ml/min; oven temp. 30°C; sample 10 ml, on-line UV and radiometric detection. Paper radioelectrophoresis was applied for qualitative and quantitative evaluation of the radiochemical purity and stability of the P149-Q[^{125}I]Y samples. The electrophoretic mobility of the samples' constituents were determined on Whatman 1 MM strips (2 cm wide, 38 cm long) in a 17 V/cm field gradient, using an electrolyte of 0.05 *M* natrium perchlorate ($NaClO_4$). The migration of radioactive bands on the strips were analysed by autoradiographic method using Radio-TLC Scanner MINI-SCAN (BIOSCAN) and Laura radio-chromatographic software.

Cross-linking assays of P149-QY peptide with receptor

For *in vitro* experiments, extracts from mammary tumours were prepared and incubated with radioiodinated P149-QY peptide in the presence of cross-linking reagent ethylene glycol-bis (succinimidyl succinate). The mixture was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12.5% SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes and analysed by autoradiography. All experiments, including tumour extract preparation and cross-linking assays of P149-Q[^{125}I]Y peptide with receptor, were carried out according to Mirowski et al [14].

Biodistribution studies of radiolabelled P149-QY peptide

The *in vivo* experiments were performed using the tumour model of transplantable mammary adenocarcinoma in C3H/W mice, obtained after subcutaneous injection of the cells suspension derived from animals bearing spontaneously growing mammary tumours (histologically classified as mammary adenocarcinoma). The tumour-bearing animals were purchased from the Department of Genetics and Laboratory Animal Breeding Cancer Centre, Institute of Oncology in Warsaw. The animals with tumour sizes ca 0.8 cm in diameter were used for biodistribution studies.

Preparations of ^{125}I-labelled P149-QY peptide were administered intravenously in doses of 0.5–1.0 MBq in 0.1 ml single injections. During the experiment, the animals were housed in metabolic cages in which urine was collected. The animals were anaesthetised and sacrificed at 30 min, 2 h and 24 h post intravenous injection (*p.i.v.*), and then selected tissues were taken out. The radioactivity of the blood pool, urine, and samples of weighted tissues and carcasses was measured using a gamma counter supplied with an adapter for whole-body measurement. The results were calculated as a percentage of the dose in organs (%ID) or a percentage of the dose per gram of tissue (%ID/g).

The animal experiments were approved by The 4th Local Animal Ethics Committee in Warsaw (authorization number ZB/3/ /2001), and were carried out in accordance with the principles of good laboratory practice (GLP).

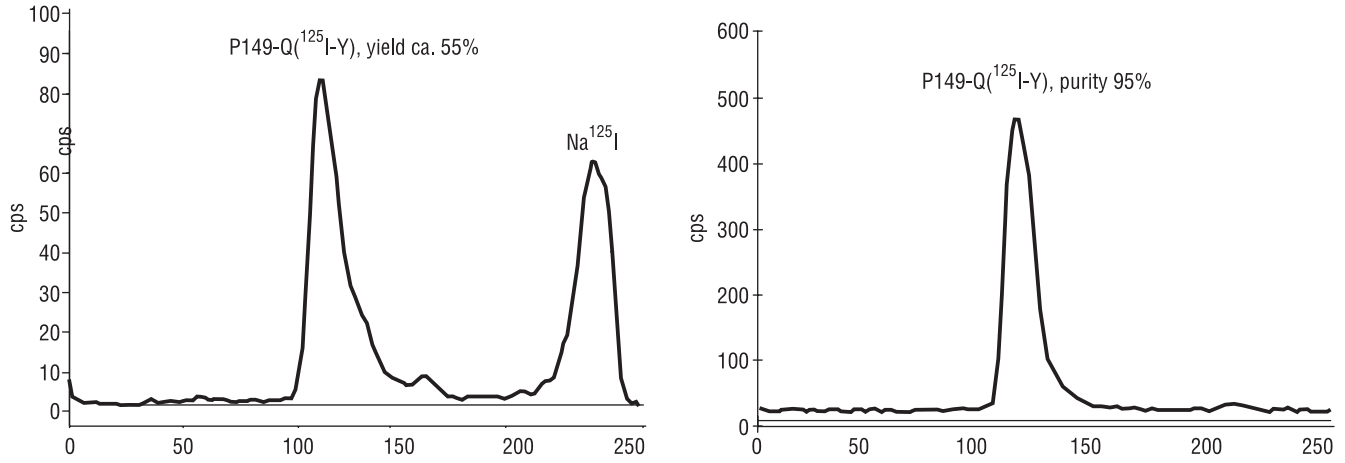

Figure 1. The electrophoregrams, on Whatman paper, of the radioiodination mixture (top), and the P149-Q[^{125}I]-Y peptide isolated by SPE C_{18} technique (bottom). X-scale: "0"— cathode, "250"— anode, "100"— point of the sample application.

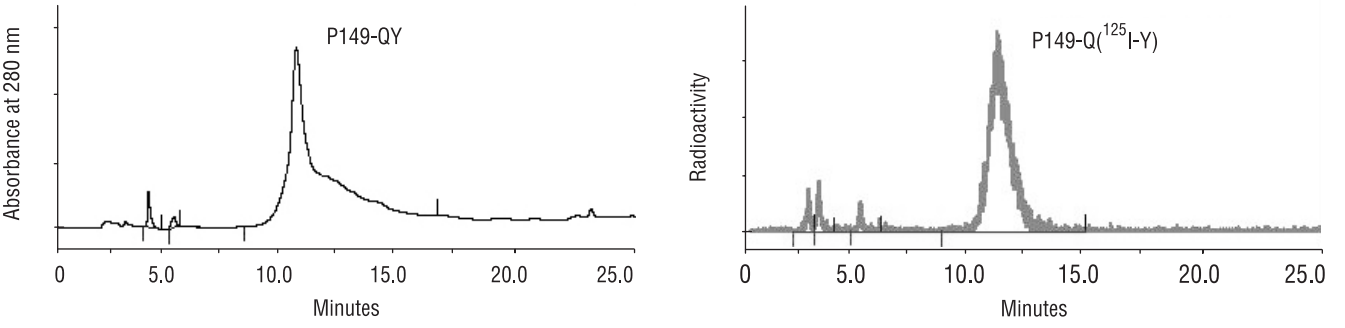

Figure 2. The HPLC chromatograms of the P149-Q[^{125}I]-Y purified on SPE C_{18}, and native peptide molecule (UV-280 nm).

Results and Discussion

Recently we have shown that the accumulation of radioiodine-labelled fetoprotein in mouse mammary adenocarcinoma was significantly higher than that in rat mammary adenoma [14]. This phenomenon is probably connected with the observation that neoplastic cells expressed receptor(s) specific for AFP. Their number per cell ranged from 2,000–300,000 [5]. This hypothesis was further confirmed by the method based on chemical cross-linking, where AFP-binding protein with molecular weight of about 30 kDa was detected in the extract prepared from mammary adenocarcinoma tissue [14].

In the present study, the 36-amino acid peptide of 90% homology to 447–480 peptide fragment of hAFP (named as P149-QY) was synthesized and radiolabelled with I-125 radionuclide. The introduction of the tyrosine amino-acid at the C-terminal end enabled preparation of the radioiodinated P149-Q[^{125}I]Y peptide with a yield of ca 50–75%. Figure 1 shows the exemplary radioelectrophoregrams of the radioiodination mixture, and the P149-Q-[^{125}I]Y) isolated and purified on SPE C_{18} cartridge. The radiochemical purity of the preparation was usually in the range 92–97% (mean 95%), and it was quite stable during storage for a few days in a refrigerator. RP HPLC analysis confirmed the identity and radiochemical purity of the radioiodinated P149-QY peptide (Figure 2).

To answer if the investigated peptide may interact with tumour cells through AFP receptor (rAFP), we used the method based on chemical cross-linking between the probable receptor and the radio-labelled ligand. Like the parent AFP molecule, the peptide fragment P149-Q[^{125}I]Y formed a complex with mouse mammary adenocarcinoma extract protein, which revealed a molecular weight of about 30 kDa (Figure 3). A similar molecular weight of radiolabelled complex was observed for AFP molecule [14], suggesting that [^{125}I]-AFP and its fragment (P149-Q-[^{125}I]Y) interact with the same protein from the mouse mammary adenocarcinoma extract. On the basis of our data, we can conclude that the interaction between AFP molecule and its receptor is possible through the amino-acid sequence present in the investigated peptide. However, further studies are needed to obtain a final answer about the length of such sequence, as well as about its binding protein present in the mammary adenocarcinoma extract.

Biodistribution studies in tumour-bearing mice have shown a higher pharmacokinetic rate of P149-Q[^{125}I]Y (Table 1) compared with the radioiodinated [^{131}I]-AFP molecule [14]. More than 40 and ca 70% of injected dose of P149-Q[^{125}I]Y were excreted with urine

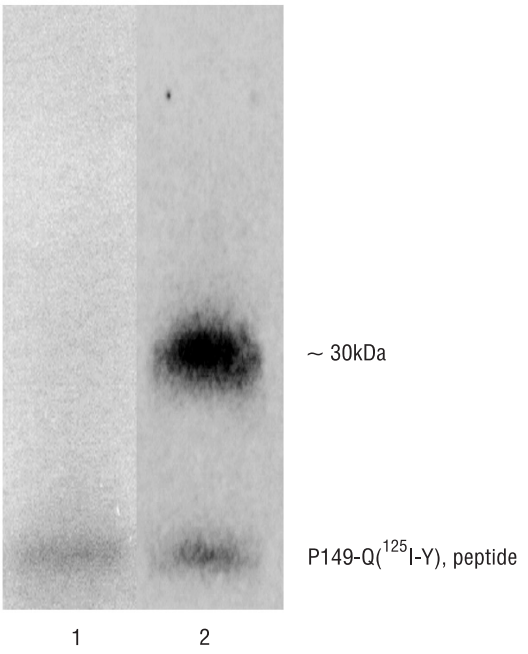

Figure 3. Autoradiographic analysis of P149-Q[^{125}I]-Y peptide before (1) and after (2) incubation with mouse mammary adenocarcinoma proteins in the presence of cross-linking reagent.

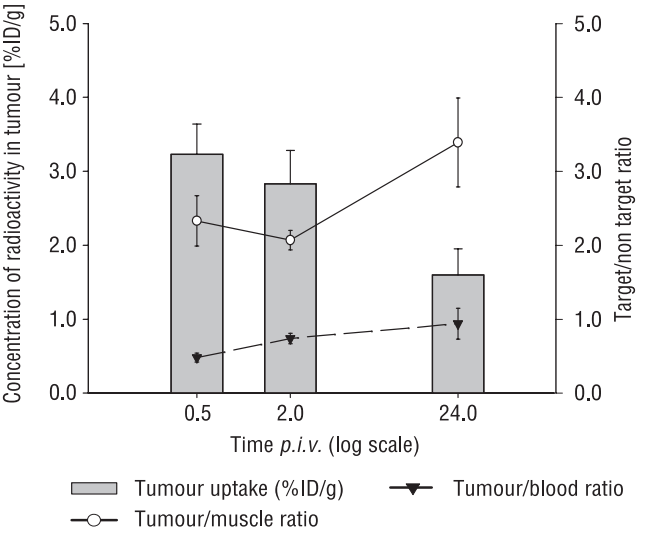

Figure 4. Variations of *in vivo* concentration of P149-Q[^{125}I]Y in mouse mammary adenocarcinoma (bars) with time after administration, in relation to ratios of the tumour/muscle (solid line) and the tumour/blood (dashed line).

Table 1. Biodistribution of P149-Q[^{125}I]-Y peptide in tumour-bearing C3H/W mice (%ID/g mean and SD; n = 6)

Organ/Tissue	0.5 h *p.i.v.*	2 h *p.i.v.*	24 h *p.i.v.*
Blood (1 ml)	6.86 ± 1.78	4.15 ± 0.37	1.65 ± 0.20
Thyroid gland	5.43 ± 1.21	6.25 ± 0.29	3.13 ± 0.65
Lung	1.78 ± 0.01	1.69 ± 0.27	2.04 ± 0.86
Liver	2.97 ± 0.62	1.68 ± 0.15	0.85 ± 0.11
Kidney	13.41 ± 1.70	12.73 ± 2.58	6.32 ± 2.49
Stomach	18.85 ± 3.44	18.60 ± 2.12	20.08 ± 4.12
Intestines	4.17 ± 0.49	3.62 ± 0.51	2.85 ± 0.19
Tumour	3.23 ± 0.41	2.83 ± 0.45	1.60 ± 0.35
Muscle	1.40 ± 0.19	1.20 ± 0.28	0.47 ± 0.02
Urine [%ID]	26.76 ± 0.98	41.10 ± 4.29	66.98 ± 7.48

2 and 24 h post administration, respectively. Relatively high accumulation in the stomach (18–20%ID/g) was observed, suggesting some instability of the radioiodinated peptide *in vivo*. However, the constant value of radioactivity retained in the stomach, likewise the decreasing concentration of activity in the thyroid gland (Table 1), do not indicate continuous release of the free radioiodine. Moderate and decreasing uptakes in the tumour tissue were observed (from 3.23 to 1.60 %ID/g after 0.5 and 24 h *p.i.v.*, respectively, Table 1). Notwithstanding, due to a faster radioactivity clearance from normal muscular tissue, and from the blood, the tumour/muscle (T/M) and the tumour/blood (T/B) ratios increased with time post administration (Figure 4), suggesting that part of the applied peptide was strongly retained in the tumour tissue. Such behaviour of the radioiodinated peptide *in vivo* may suggest the specific mechanism of its binding and retention in the tumour cells, probably due to ligand-receptor interaction, and promises its better characteristic as a potential *in vivo* tracer for scintigraphy of tumours in comparison to the whole radiolabelled AFP glycoprotein.

The two cysteines present in P149 peptide may favour, in solution, the intrapeptide disulfide bond formation [22, 23] that leads to loss of anti-growth activity of the peptide. MacColl et al [23] observed also that the presence of some ions may influence the peptide activity e.g. Zn(II) ion binds to P149 peptide, stabilising its active form but Co(II) ion acts in reverse, catalysing the loss of peptide activity. Therefore, some efforts should be made to prevent formation of the inactive form of the peptide in the radiolabelling process, or a new peptide analogue should be synthesised, e.g. from which the two cysteine residues would be replaced by two alanines. The 2 Cys = > 2Ala replacement analogue cannot form a disulfide bond. Recently, it was shown that such a peptide was active in the inhibition of the growth of prostate and breast cancer cell lines, as well as against breast cancer *in vivo* [24, 25].

In conclusion, our study shows that radioiodinated P149-QY peptide reveals some positive features as the AFP receptor radioligand. However, some additional structural modifications of the initial peptide molecule may perhaps improve avidity of the peptide with the receptor.

Acknowledgements

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