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ORIGINAL ARTICLE

Effective treatment of pancreatic neuroendocrine tumours transfected with the sodium iodide symporter gene by ^{186}Re-perrhenate in mice

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Abstract
Purpose ReO_4^- has similar kinetics regarding the sodium iodide symporter (NIS) to I^- and TcO_4^- in NIS-expressing tissue. We investigated the therapeutic potential of $^{186}ReO_4^-$ in NIS-transfected neuroendocrine tumour tissue.
Methods For experiments, the stably NIS-transfected pancreatic neuroendocrine cancer cell line Bon1C was used. NIS-mediated internalization and externalization experiments in vitro and a biodistribution study in nude mice bearing Bon1C xenografts were performed. A therapy study was also conducted consecutively in nude mice xenografted with Bon1C in which the mice were injected intravenously with $Na^{186}ReO_4$.
Results In vitro studies showed exponential internalization and efflux kinetics of $^{186}ReO_4^-$ in the cell line. The biodistribution study showed high uptake of $^{186}ReO_4^-$ in NIS-expressing tumours. Tumour growth inhibition was significant after injection of $^{186}ReO_4$ in two groups of animals treated with activity levels below the determined maximum tolerable activity as compared to controls.
Conclusion These results indicate that the use of $^{186}ReO_4^-$ in the treatment of NIS-expressing neuroendocrine tumours is feasible and support the concept of using NIS as a therapeutic target for $^{186}ReO_4^-$.

Keywords Sodium iodide symporter · ^{186}Re-Perrhenate · Rhenium-186 · Neuroendocrine tumours · Radionuclide therapy

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Introduction

Frequently, gastrointestinal and pancreatic neuroendocrine tumours are detected at a very advanced stage. This is due to the general absence of symptoms in the early, still localized stage when surgery alone may be curable. Most neuroendocrine tumours produce peptide hormones including serotonin, gastrin, insulin, vasoactive intestinal peptide, pancreatic polypeptide and substance P, which are catabolized mostly by a first-pass effect in the liver before they can cause systemic symptoms. In the majority of cases, the liver is the first location for metastases from intraabdominal primary tumours. Once the tumour has metastasized into the liver, the secreted endocrine active substances can reach the systemic circulation and cause symptoms. At a metastasized stage, palliative treatment including tumour debulking, liver artery embolization, cytoreductive medication and endocrine therapy with synthetic somatostatin analogues are the current options. It is known that 80–100% of neuroendocrine tumours produce chromogranin A as a specific tumour marker [1–5].

Over recent years, several studies of the structure and function of the sodium iodide symporter (NIS) and its role in the therapy of malignant diseases have been published. Such studies became possible with the cloning of the rat

and human forms of this molecule in 1996 [6]. It has been shown that NIS, after transfection, is a target for radioiodide therapy [6, 7]. The transport of two Na^+ cations in exchange for one I^- anion by the basolateral transmembrane NIS follows the electrochemical gradient created actively by Na^+/K^+-ATPase [8]. NIS not only transports iodide but also negatively charged ions with a similar ionic radius, such as $^{99m}TcO_4^-$ and $^{186}ReO_4^-$ anions [9–12]. These anions are taken up by the thyroid gland but do not take part in the synthesis of thyroid hormones since they are not subject to organification.

NIS has been used for decades in the diagnosis and therapy of thyroid diseases, e.g. in the treatment of hyperthyroidism and staging and therapy of thyroid carcinoma. In differentiated thyroid tumours, radioiodide therapy is an important routinely performed treatment to eradicate thyroid remnants after surgery as well as to detect and treat metastases [13]. This procedure leads to relapse-free 5-year survival rates of more than 90%. NIS expression has also been detected in extrathyroid tissue including the stomach, salivary glands, lactating breast and potentially even breast cancer [14–16].

Transfection of NIS-containing vectors into non-NIS-expressing malignancies, followed by radioiodide therapy is an interesting option. The NIS gene has been transfected into various tumour tissues. Several studies have shown that the internalization of radioactive iodide in these cells is up to 225-fold higher than in control cells [17–20]. A therapeutic effect in vivo was demonstrated for the first time by Spitzweg et al. [21], and more recently by our own group [22].

In recent studies, we have demonstrated promising therapeutic results with $^{131}I^-$ and $^{99m}TcO_4^-$ [22], and with respect to the results of Dadachova et al. and Zuckier et al. [12, 23], the aim of this study was to investigate the ability of $^{186}ReO_4^-$ to treat NIS-transfected neuroendocrine tumours in vivo.

Materials and methods

Cell lines

The serotonin-secreting and chromogranin A-expressing neuroendocrine pancreatic carcinoid cell line Bon1 was stably transfected with a plasmid bearing the human NIS gene and a geniticin resistance gene. Expression of the hNIS gene is controlled by the chromogranin A promoter. This transfected cell line was renamed Bon1C (pcDNA 3.1-CgA-NIS), as described previously [24]. NIS expression was proven by RT-PCR, Western blotting and repeated functional assays. The ability to take up $^{186}ReO_4^-$ was tested in in vitro internalization and externalization studies. Internalization and externalization kinetics of $^{186}ReO_4^-$ were similar to those of $^{131}I^-$ and $^{99m}TcO_4^-$, confirming the findings of Zuckier et al. [23].

Cell culture

Bon1C cells were cultured in Dulbecco's MEM/Nut mix F12 medium (Gibco BRL, Karlsruhe, Germany) supplemented with L-glutamine, 10% fetal calf serum (Gibco BRL), 100,000 IU/l penicillin, 100 mg/l streptomycin and 400 μg/ml geniticin. Cells were grown at 37°C in an atmosphere of 95% air/5% CO_2.

Kinetics of $^{186}ReO_4^-$ uptake

Approximately $4x10^4$ cells were incubated with 185 kBq/ml $^{186}ReO_4^-$ in 1 ml Hank's balanced salt solution (HBSS) with and without 1 *M* perchlorate. After 60 min they were washed twice with ice-cold HBSS, lysed in 1 *M* KOH, and counted using a Cobra II auto-gamma gamma counter (Packard BioScience, Dreieich, Germany) in a 100–180 keV window (γ energy of $^{186}ReO_4$ about 137 keV). For the internalization studies, $4x10^4$ cells were incubated with 185 kBq/ml $^{186}ReO_4^-$ in 1 ml HBSS for 5, 10, 15, 30, 45, 60, 90 and 120 min [22]. Efflux of $^{186}ReO_4^-$ was investigated by incubating $4x10^4$ cells with 185 kBq/ml $^{186}ReO_4^-$ in 1 ml HBSS for 60 min. Cells were washed twice with ice-cold HBSS and incubated with HBSS for 5, 10, 15, 30, 45, 60, 90 and 120 min before lysis with KOH. Experiments were performed in triplicate [22].

Animal experiments

Animal experiments were conducted in accordance with German law and were reviewed and approved by the local animal protection committee. Athymic nude (Nrc nu/nu) mice at 6–8 weeks of age were obtained from Charles River (Sulzfeld, Germany). The animals were housed under pathogen-free conditions with a 12-h light/12-h dark schedule and fed autoclaved standard food and water ad libitum. For xenografts, $6x10^6$ tumour cells in culture medium were injected into the flanks of the mice.

In vivo biodistribution

Animals were injected with 370 kBq of $^{186}ReO_4^-$ in 0.1 ml 0.9% saline solution into a tail vein. For the biodistribution studies $^{186}ReO_4^-$ was obtained from Covidien Deutschland. Three mice were killed at 30 min, 60 min, 120 min, 360 min, 24 h and 48 h after injection. Tumour, thyroid gland, salivary glands, stomach, liver, kidneys, small intestine, spleen, pancreas, lung, heart, muscle and bone were immediately carefully removed, weighed on an

analytic scale and their γ activity counted using a Cobra II auto-gamma gamma counter (Packard BioScience) in a 100–180 keV window. Uptake is expressed as percentage of injected dose per gram (%ID/g) tissue, related to a reference standard of the injected activity.

Radioisotope therapy studies

Nude mice bearing Bon1C xenografts were randomized into six groups after tumour sizes reached 260±30 mm^3. Treatment groups were injected with 37 (n=5), 111 (n=5), 185 (n=5), 259 (n=8) and 370 MBq (n=8) $^{186}ReO_4^-$ (Covidien Deutschland) once into a tail vein. Both tumour volume, measured using a microcalliper (volume of a rotation ellipsoid was assumed as height × length × width × 0.5), and body weight were recorded weekly. Mice were killed if their weight loss was more than 30%, or if they showed cachexia and/or signs of infection or weakness, or tumour ulceration or haemorrhagia. In all cases, the study was terminated after 7 weeks.

Statistical analysis

For statistical analysis the software SigmaStat was used. The data were analysed using one-factorial ANOVA. Pair-wise post hoc comparisons were conducted when necessary using Bonferroni tests. For all statistical analyses, p values less than 0.05 were deemed significant. In the figures, values are means, and error bars indicate the standard error of the means (SE).

Results

Kinetics of $^{186}ReO_4^-$ uptake

The Bon1C cell line showed intense uptake of $^{186}ReO_4^-$. Uptake inhibition by adding perchlorate was tested and proven in the first routine uptake experiments with a 60-min incubation time as described above. The uptake was eightfold higher than in perchlorate-inhibited control cells (data not shown). Internalization was fast, reaching a plateau after 15 min (Fig. 1a). Externalization studies showed a rapid efflux of $^{186}ReO_4^-$ from Bon1C cells with an approximate effective half-life of 5 min (Fig. 1b).

In vivo biodistribution

In this study we investigated the biodistribution and therapeutic value of $^{186}ReO_4^-$ in nude mice bearing the NIS-expressing Bon1C tumour. As expected, $^{186}ReO_4^-$ showed the highest uptake in the thyroid gland. The maximum was reached after 30 min (385±98.3 %ID/g; Fig. 2c). The second highest uptake was in the stomach, also reached after 30 min (18.4±4.4 %ID/g; Fig. 2a). It is remarkable that Bon1C tumour showed a maximum uptake at 120 min and showed the third-highest uptake of all measured organs (9.1±1 %ID/g; Fig. 2b) followed by the salivary glands (6.1±2.7 %ID/g), which showed the highest uptake again after 30 min. The other organs counted showed negligible uptakes due to a lower or no expression of NIS in these organs (Fig. 2a).

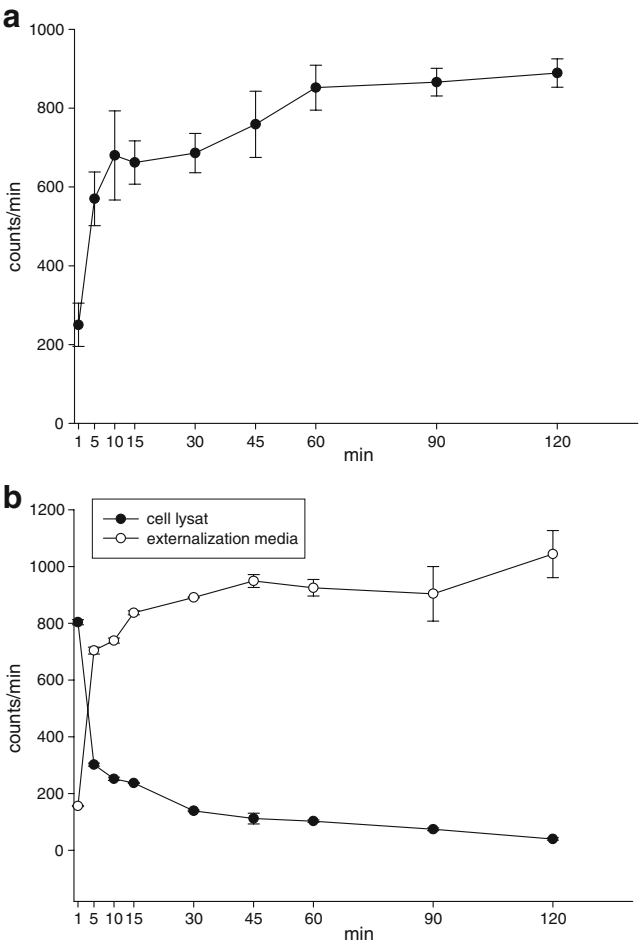

Fig. 1 Kinetics of $^{186}ReO_4^-$ uptake in 4x10^4 stably NIS-transfected Bon1C cells (*error bars* SE). **a** internalization of $^{186}ReO_4^-$ into Bon1C cells. **b** Efflux of $^{186}ReO_4^-$ from Bon1C cells (*externalization media* initially not radioactive HBSS to measure externalization of radioisotope)

The characteristics of the %ID/g curves over time were very similar for each organ except the curve for the tumour. This also means that the thyroid gland did not retain $^{186}ReO_4^-$, and thus externalizes in the same exponential way as the other organs (Fig. 2c). In contrast, the tumour first showed increasing uptake reaching a maximum after 2 h and subsequently showed less rapid efflux than other organs up to the 24-h time-point (Fig. 2b). After 48 h, every

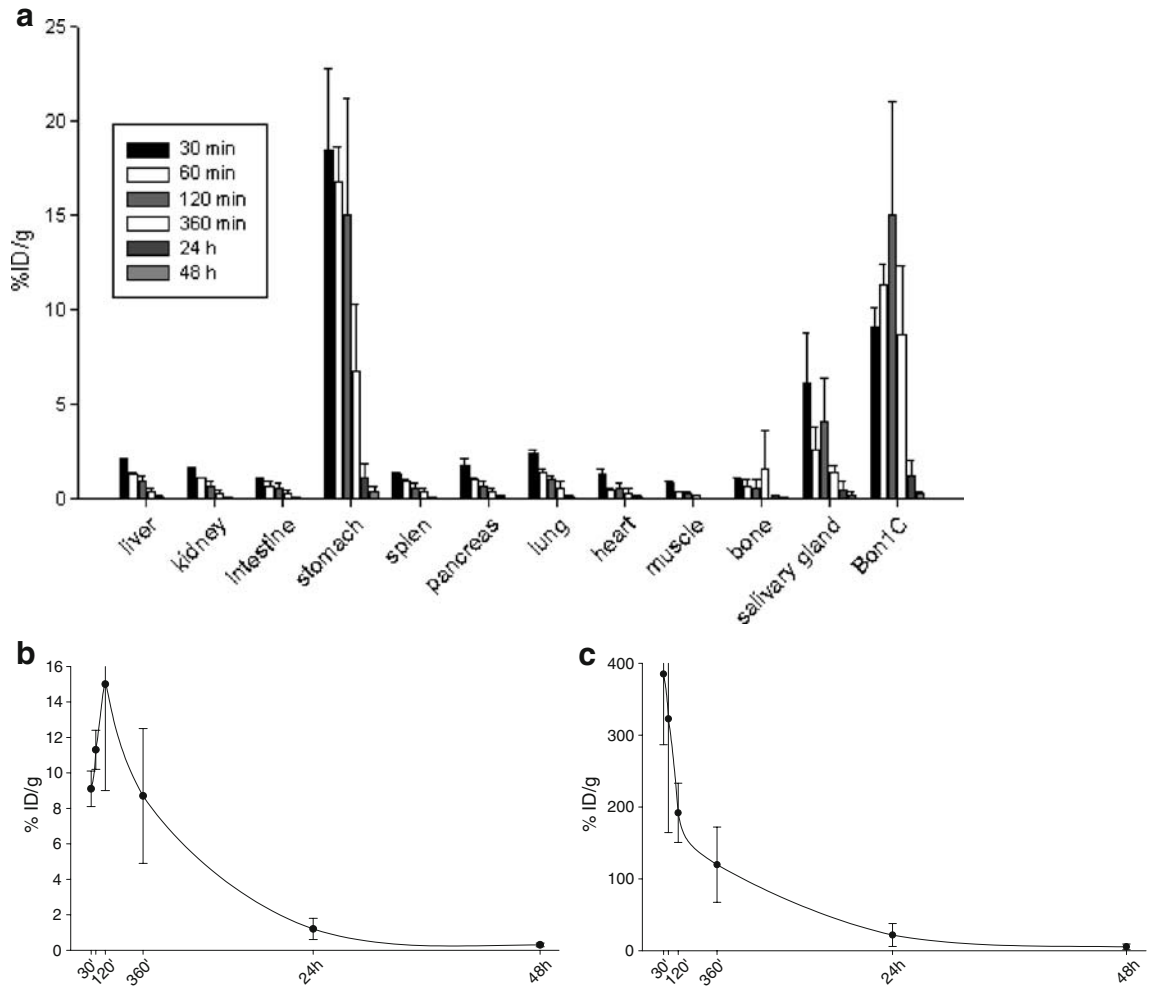

Fig. 2 Biodistribution of $^{186}ReO_4^-$ in nude mice bearing NIS-expressing Bon1C tumours. **a** Represented are all explanted organs except the thyroid. **b** Biodistribution of $^{186}ReO_4^-$ in Bon1C tumours over time. **c** Biodistribution of $^{186}ReO_4^-$ in the thyroid over time (*error bars* SE)

organ showed a %ID/g less than 0.5 except the thyroid gland, in which 5.5±3.9 %ID/g remained due to the high uptake at the beginning of the experiment.

Radioisotope therapy studies

In the therapy study with different activities of $^{186}ReO_4^-$ the maximum tolerable activity in nude mice was considered to be 185 MBq per animal. Tumour growth inhibition and even tumour volume reduction was observed in animals treated with 111, 185, 259 and 370 MBq (Fig. 3). In animals treated with 37 MBq $^{186}ReO_4^-$, no significant difference in tumour growth compared to the control group was seen. In animals treated with 111 MBq, tumour growth inhibition was significant ($p<0.05$) as compared to growth in the control group on days 21 and 28. In animals treated with 185 MBq $^{186}ReO_4^-$ tumour volume reduction was significant ($p<0.05$) as compared to the volume in control group on day 21 and remained significant until day 42 of

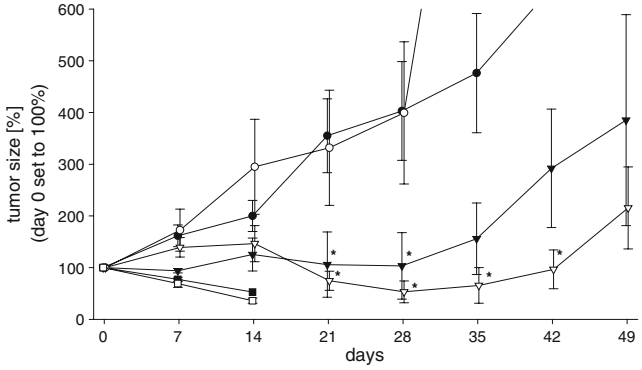

Fig. 3 Tumour growth inhibition of Bon1C NIS-expressing neuroendocrine pancreas carcinoma xenografted into nude mice following treatment with different activities of $^{186}ReO_4^-$ (37, 111, 185, 259, 370 MBq). The radioisotope was injected once into a tail vein (*error bars* SE; *$p<0.05$)

the study. The activity of 185 MBq $^{186}ReO_4^-$ per mouse was the most effective dose in this study concerning the duration of tumour growth inhibition with a tolerable toxicity. Tumours of animals treated with 259 MBq or 370 MBq $^{186}ReO_4^-$ showed the greatest volume reduction. The activities of 259 MBq and 370 Bq caused severe side effects due to radiation injury which were lethal or fulfilled the criteria for removal of the mice from the study. All animals in these groups died or had to be killed in the first 14 days of the study. These animals showed general weakness and respiratory problems. The macroscopic appearance of the explanted stomach showed signs of radiation gastritis. Weight loss of the mice increased dose dependently: 2.9±1.2%, 4±2.5%, 15.6±12.5%, 14.2±8.1% and 29.2%±11.2% in animals treated with 37, 111, 185, 259 and 370 MBq, respectively.

Discussion

The stably transfected NIS-expressing cell line Bon1C in which NIS expression is controlled by the chromogranin A promoter accumulates $^{186}ReO_4^-$ effectively. Chromogranin A is produced by neuroendocrine tissue as well as by 80–100% of neuroendocrine tumours and is a specific tumour marker [2–4]. The uptake of $^{186}ReO_4^-$ was eightfold higher than in control cells in vitro. This fits the data for ^{131}I and $^{99m}TcO_4^-$, where the uptake is 52-fold and 18-fold higher, respectively. Internalization and externalization kinetics were rapid and similar to the values found in studies performed with $^{131}I^-$ and $^{99m}TcO_4^-$ [22]. Further studies, which are already in progress, are seeking to elucidate the possibility of extending the intratumoral radioisotope half-life in the therapeutic setting, e.g. by reducing efflux through lithium administration and anion transporter inhibitors [25, 26].

In vivo the NIS-expressing neuroendocrine Bon1C tumours showed strong uptake of $^{186}ReO_4^-$. As compared to $^{131}I^-$ and $^{99m}TcO_4^-$ (36.1±26.7 %ID/g; 24.6±24.0 %ID/g), the tumour accumulated 15±6 %ID/g $^{186}ReO_4^-$ at peak activity levels. As seen previously, and also previously described by other groups [27–29], the externalization of the radiopharmaceutical out of the tumour was fast. The peak activity was found in every tissue at 30 min with the exception of tumour tissue. In tumour tissue the maximum uptake was reached after 120 min. The prolongation of uptake compared to in vitro experiments can be explained by different geometries regarding the monolayer cell culture and the three-dimensional tumour. Externalized radioisotopes in vitro diffuse off into the incubation medium. In vivo, however, contiguous cells again take up previously externalized anions which correlates with high levels of NIS-expressing cells as well [30]. Besides the thyroid gland, the stomach and salivary glands also strongly internalize radioisotopes due to expression of NIS in these tissues [31–34]. Therefore, toxicity to these organs must be kept in mind for therapeutic studies. The thyroid gland especially is at risk because of the very high uptake and/or accumulation (iodide isotopes) of isotopes transported by NIS. Nevertheless, using isotopes which take no part in the synthesis of thyroid hormones therefore reducing the exposure time of this organ seems to be a valuable approach to reducing radiation injury [12, 35, 36].

We chose $^{186}ReO_4^-$ instead of $^{188}ReO_4^-$ because of the lower β-energy (1.1 MeV vs. 2.1 MeV) of $^{186}ReO_4^-$. We expected lower toxicity because of the lower range of emitted electrons. In the therapy study with $^{186}ReO_4^-$ significant tumour growth inhibition and even tumour volume reduction were seen in animals treated with 111 MBq and 185 MBq compared to the control group ($p<0.05$). Mice treated with 37 MBq showed no significant difference in tumour growth compared to the control group. Mice treated with 259 MBq and 370 MBq showed a reduction in tumour volume but all mice in these two groups had to be killed 14 days after injection at the latest because of severe side effects fulfilling the stop criteria. Of the treated animals with NIS-expressing neuroendocrine tumours, 71% showed remission, 6.5% stable disease and 22.5% progressive disease over all groups treated with different activity levels. Of the animals treated with tolerable activities of $^{186}ReO_4^-$ for nude mice (37, 111 and 185 MBq), 53.3% showed regression. The maximum tolerable activity for nude mice is considered to be 185 MBq per animal. In view of the general weakness and respiratory problems of the animals and the macroscopic appearance of the explanted stomach, we consider that the toxicity of 259 MBq and 370 MBq could have been due to myelosuppressive effects of radiation and digestion problems caused by radiation gastritis.

Following our previous results regarding $^{99m}TcO_4^-$ and $^{131}I^-$ [22] showing therapeutic efficacy of these isotopes in NIS-transfected neuroendocrine Bon1C xenografts, we have now demonstrated that NIS-expressing neuroendocrine tumours can at least in principle be treated successfully and effectively using $^{186}ReO_4^-$.

For clinically suitable therapy options an appropriate vector would be needed. Further studies should focus on in vivo transfections of hNIS controlled and cotransfected with a tissue-specific promoter (CgA) with, for example, adenoviruses as vectors.

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