Action and Efficacy of p-[131I]Iodo-L-phenylalanine on Primary Human Glioma Cell Cultures and Rats with C6-gliomas

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Abstract. Background: Recently we demonstrated high selectivity and long retention of p-[125I]Iodo-L-phenylalanine (IPA-123) for diagnostic purposes in gliomas. Here we describe the potency of the iodine-131-labelled analogue p-[131I]Iodo-L-phenylalanine (IPA-131) for experimental treatment of gliomas. Materials and Methods: Two human glioma cell cultures and 15/20 rats with C6-gliomas were exposed to IPA-131. Results: After 24-h-exposure the cell number of the glioma cell cultures was reduced by 77% and 79%, respectively. Four out of five untreated rats died 0-24 days after implantation and had verifiable tumours. Seven animals died despite therapy, while 8 were soon healthier, gained body weight and were sacrificed 81 days after implantation, at which time survival of the treated animals was significantly prolonged (p<0.05). At autopsy, instead of tumours there were substance defects, surrounded by pre-existing parenchyma infiltrated by probable tumour cells that were not discernible from reactive astrocytes, lymphocytes, monocytes and multinuclear giant cells. Conclusion: IPA-131 has promising activity against human glioma cells in vitro and experimental gliomas in vivo.

Malignant gliomas are among the least understood and most incurable malignancies. They manifest with a peak incidence between 45 and 70 years old, but may manifest at any age (1). Even though aggressive surgical therapy in combination with radiochemotherapy can prolong life, or improve the quality of life of individual patients for a certain period of time, the overall survival rate has remained virtually unchanged for decades (1, 2). In order to overcome these dismal prospects, various experimental therapies are administered, among them the use of death ligands, methods for sensitising glioma cells to the induction of apoptosis, p53 gene transfer, photodynamic therapy with 5-aminolevulinic acid, approaches to target the expression of therapeutic genes selectively to tumour cells, or aimed at different targets like the coagulation system, to name only a few (3-9).

In recent studies we demonstrated the effectiveness and safety of single photon emission tomography (SPET) with p-[123I]Iodo-L-phenylalanine (IPA-123) for brain tumour imaging, not only in the experimental C6 glioma model but also in patients harbouring diffuse gliomas (10, 11). IPA-123 crosses the blood-brain barrier after intravenous administration and accumulates specifically in malignant gliomas with marked retention in neoplastic cells. We postulated that L-phenylalanine conjugated to the beta- or alpha-emitting isotopes iodine-131, iodine-125 or astatine-211 (12-14) could represent a new tool by which an effective radiation dose can be concentrated on the tumour cells. In consequence, we developed the iodine-131-labelled phenylalanine p-[131I]Iodo-L-phenylalanine (IPA-131) by an improved one-step preparation. The purpose of this work was to assess the effectiveness and the morphological changes caused by IPA-131 on primary human glioma cell preparations and orthotopic C6 gliomas in rats.

Materials and Methods

4-Bromo-L-phenylalanine, used as starting material for the radioiodination, was purchased from Sigma-Aldrich (Deisenhofen, Germany). Physiological 0.9% NaCl and PBS (Braun Melsungen, Germany) were obtained from the local hospital pharmacy. Na2S2O4 and Cu(II) sulphate were from Merck (Darmstadt, Germany). Unless stated otherwise, all other chemicals and solvents were of analytical grade and obtained via our local hospital pharmacy. Sodium [131I]iodide for radiolabelling was obtained in the highest obtainable radiochemical purity, generally 180-500 MBq in 10-20 µl PBS solution from Tyco International (Petten, Netherlands). HPLC purification was performed on a Hewlett Packard HPLC system consisting of a binary gradient

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pump (HP 1100), a Valco 6-port valve with 250 ml loop, a variable wavelength detector (HP 1100) with a UV detection at 254 nm and a sodium iodide scintillation detector (Berthold, Wildbad, Germany), using reversed-phased column (250 x 4 mm, Nucleosil-100, Latex, Eppelheim, Germany). The column was eluted at a flow rate of 1.2 ml/min with water/ethanol/acetic acid (85:10:1, v/v).

Preparation of p-[131]iodo-L-phenylalanine. p-[131]iodo-L-phenylalanine (IPA-131) was prepared by non-isotopic Cu(II)-assisted [131]iodo-debromination of p-bromo-L-phenylalanine in the presence of ascorbic acid. In detail, a mixture of sodium [131]iodide (185-370 MBq in 20 - 50 µl 0.1 N NaOH) and 3 µl aqueous Na2S2O4 (4.6 mg Na2S2O4/ml) was evaporated to dryness by passing a stream of nitrogen through a reaction vessel at 100°C, followed by addition of 100 µl of p-bromo-L-phenylalanine hydrobromide (0.25 mg/ml 0.1 N H2PO4), 20 µl aqueous L-ascorbic acid (10 mg/ml) and 10 ml aqueous Cu(II) sulphate (0.1 mol/l). The reaction vessel was heated for 60 min at 160°C in a heating module (Fispec), cooled and the mixture diluted with 150 µl water. The radioiodinated product was separated from unreacted starting materials and radioactive impurities by HPLC. Generally IPA-131 was obtained in 88 ± 5% radiochemical yield. The fraction containing IPA-131 (retention time 19.5 min) was collected into a sterile tube, buffered with 0.5 M PP (pH 7.0, Braun, Melsungen, Germany), diluted with physiological saline and sterile filtered through a 0.22-µm sterile membrane (Millipore, Millipore, Molsheim, France) to an isotonic and injectable radiochemical for in vitro and in vivo studies.

Cell cultures. The human glioma cell cultures HOM-Tx 3686 and HOM-T 5135 (both from primary human glioblastoma multiforme) were provided by the Institute of Human Genetics, University of the Saarland (Homburg, Germany). Cells were cultivated in RPMI-1640 medium and Dulbecco’s modified Eagle medium (sodium pyruvate-free, supplemented with L-glucose and pyridoxine, respectively), supplemented with 10% heat-inactivated foetal calf serum (FCS), penicillin (50 U/ml), streptomycin (50 µg/ml) and 50 µl insulin (PromoCell, Heidelberg, Germany). In order to achieve the morphological characteristics of glioma cells, they were grown on standard glass slides. After development of a confluent lawn of cells, the cultures were exposed to (0.001-10 µCi/ml IPA-131 for 24 h at 37°C 5% CO2). Then the medium was removed and the cells were fixed in 4% neutral buffered formalin.

Magnetic resonance imaging. In 10 randomly-chosen animals, the presence of the tumour was confirmed by cranial MRI. Therefore, the animals were anaesthetised with chloral hydrate (250 mg/kg, i.v.). Magnetic resonance imaging was performed 9 days after tumour implantation (before IPA-131 injection) and after the second IPA-131 injection on day 18 as well as on day 81 before sacrifice, using a 2.4-Tesla small animal magnetic resonance tomograph (Bruker Biospec 2.4, Karlsruhe, Germany). This system was equipped with a rat coil to hold the animal and transmit homogeneous signals. All rats were imaged while under anaesthesia. Multiple axial and coronal images (T1- and T2-weighted) were acquired for 20 min without contrast media. The parameters used were: T1-weighted (TR = 100 msec, TE = 6.5 msec, flip angle 30°, FOV = 2x2 cm, 256 x 128 x 64 matrix) and T2-weighted (TR = 500 msec, TE = 17.5 msec, 1 acquisition, RARE factor 16, FOV = 2x2 cm, 256 x 256 x 32 matrix, slice thickness 16 mm).

Morphological examination. HOM-Tx 3686 and HOM-T 5135 cells grown on standard glass slides were fixed in 4% neutral buffered formalin and stained with the Giemsa method. The cell number was calculated in 10 consecutive high-power fields (x 40).

At autopsy of the rats, besides the brains, other organs were harvested including the heart, lungs, liver, spleen, kidney, skin and colon. The brains were cut in coronal slices of about 2-3 mm thickness. All tissues from the animals were fixed in 4% neutral buffered formalin and embedded in paraffin wax. Sections were stained with haematoxylin-eosin and Verhoeff-van Gieson and examined histopathologically.

Results

In vitro studies. After 24-hour-exposure of the primary human glioma cell lines with IPA-131, the cell count was clearly reduced (Figure 1a, 1b). HOM-Tx 3686 showed a 77%, and HOM-T 5135 a 79% lower cell count after exposure to 1 µCi/ml IPA-131 compared to parallel grown cell cultures without exposure. The surviving cells contained only sparse cytoplasm, the nuclei were shrunk and contained chromatin.

Survival of rats. Successful tumour implantation was observable clinically. All animals but one became severely ill with progressive hemiparesis, conspicuous behavioural and vigilance disturbances. IPA-131-treated animals survived longer than animals of the control group (Figure 2). In eight treated rats signs and symptoms related to the space occupying lesions improved quickly after initiation of the therapy; the rats soon appeared more healthy and gained body weight. They were sacrificed 81 days after implantation at which time Kaplan-Meier survival curves showed significantly longer survival of the treated animals group (p < 0.05), and no tumour was demonstrable with MRI. In the treated animals no toxic effects were observed clinically. Seven animals died despite treatment 18-29 days after implantation. At autopsy all of these animals had large tumours. Four animals died during anaesthesia for a MRI
Figure 1. Morphological features. Cell culture before (a) and after treatment with 1 μg/ml IPA-131; there is a great reduction in cell number and the remaining cells are small with sparse cytoplasm and condensed chromatin (b) (Giemsa stain, original magnification x200). T1-weighted MRI of rat with C6-glioma: even though the tumour does not contrast normal brain parenchyma, the space-occupying effect is observable as there is no subarachnoid space or ventricle visible (c). Histomorphologically, besides large central tumours, tumour is often also attached to the calotte and subgaleal (d, H&E-stain, original magnification x20). The tumour often grows along the ventricles (e, H&E-stain, original magnification x20). T1-weighted MRI 9 days after tumour implantation (f), and after treatment, shortly before euthanasia, shows a large hypointense area at the site where the tumour was. Furthermore, the subgaleal tumour disappeared (g). The same brain at autopsy after formalin fixation harbours a great defect (h). Low-power view of site of previous tumour implantation (i, H&E-stain, original magnification x10); at higher magnification there are lymphocytes, monocytes, multinuclear giant cells and calcifications (j). Some of the cells might either represent residual glioma cells or reactive astrocytes (k, H&E-stain, original magnification x400).
Figure 2. Graphic illustration of survival of untreated (grey) and IPA-131-treated (black) rats with C6-gliomas according to the Kaplan-Meier method. The y co-ordinate shows surviving rats in percent, the x co-ordinate shows the time in days.

Despite great advances regarding diagnostics and the therapy of diffuse gliomas, the prognosis of patients with this cancer is still poor. The nature of this invasive process determines that the diseased tissue is usually a composition of tumour-, reactive- and pre-existing- cells, among them the neurones. Not even the smallest surgical instruments are fine enough to bypass the pre-existing neurones in order to resect only the invasive tumour cells. More promising should be a therapy by which a substance is applied systematically that selectively accumulates in tumour cells and produces a toxic effect or uses the immune system to selectively destroy the tumour cells.

Because brain tumours share the ability to accumulate different amino acids more effectively than normal tissues and any pathology other than tumour, imaging with radiolabelled amino acids has become a promising tool for studying brain tumours (17, 18, 19). As shown in previous investigations, the iodine-123-labelled amino acid IPA-123 accumulated highly and specifically in experimental and human gliomas (10, 11, 20). Since radiotherapy is the treatment typically used in patients diagnosed with brain tumours (21), we undertook the present study to document whether the beta radiation-emitting iodine-131 analogue IPA-131 has the potential for a selective regional radiotherapy for diffuse gliomas which can be administered systemically.

First, we tested the effect of IPA-131 on human glioma cells. Two human glioma cell cultures were exposed to IPA-131 in vitro, resulting in an evident reduction of tumour cells. Cytologically, the mode of cell death was apoptosis since the remaining tumour cells contained only sparse cytoplasm and apoptotic bodies; in other cells, the nuclei were shrunken and contained condensed chromatin.
In the animal model, the survival time of treated rats with C6-gliomas was significantly increased. Moreover, our data would have been even more promising, if one discounted the one long-term surviving untreated animal which had no evidence of successful tumour implantation, a relatively rare occurrence. In the other animals, successful tumour implantation was obvious clinically and by MRI studies. The quality of life improved quickly in a substantial number of the treated rats. Only symptoms caused by the tumours themselves were observed. There were no serious adverse therapy-related events. Surprisingly, no treated animal died after the 29th day. One explanation would be that some rats are sensitive to the therapy while others are not. From our clinical observations, we tend to assume that the deceased animals were treated too late. The relevant animals were already severely ill with neurological deficits, conspicuous behavioural and vigilance disturbances. Most probably the tumours were already too large and the therapy-induced reactions (i.e. oedema) increased the space-occupying effect and the intracranial pressure, leading to death. Four of these animals died during anaesthesia for the second MRI-scan which posed additional stress on these animals.

In the other animals, the use of the MRI-scan for follow-up was very helpful because successful tumour implantation could be demonstrated and, later, the defects of the previously implanted gliomas were obvious in the successfully treated animals. Even though the intracranial tumours were usually not discernible from the surrounding brain tissue, the extracranial tumours were clearly visible and allowed easy monitoring of the space-occupying lesions.

At autopsy in long-term surviving rats there was only minimal evidence of residual disease. We only observed increased cellularity with astrocytic cells. Most probably these cells represent surviving tumour cells, however, morphologically these cells were not distinguishable from reactive astrocytes. Immunostaining with antibodies directed against glial fibrillary acidic protein or the evaluation of proliferating cells with Ki67-labelling was not helpful in this question (data not shown). To the best of our knowledge, there is no reliable test available to differentiate reactive astrocytes from potential residual C6-tumour cells if the morphology of the cells does not help.

Another observation at autopsy was that, compared to rats without a tumour, all successfully treated animals had enlarged ventricles. This observation can be explained by two mechanisms. In rats with C6-gliomas, the tumour cells do not grow only at the site of implantation, but they also spread along the subarachnoid space and along the ventricular walls. So, in the first place, they lead to a disturbance of cerebrospinal fluid (CSF) flow. Secondly, the tumour cells exert a direct space-occupying effect when they grow in the ventricles and in the subarachnoid space. After elimination of the tumour cells by IPA-131, the enlarged CSF space remains.

One discussed problem of systemically administered IPA-131 consists of the radiation dose which does not reach the target cells, but the whole body and susceptible organs like bone marrow, the liver, the urogenital system and, of course, the thyroid gland. Liver and kidneys of the treated animals did not show any histomorphological abnormality and no animal had clinical signs of malfunction of any organ as no side-effects were observed clinically. No seizures were observed. In addition, the radiation doses, estimated based on previous biodistribution studies in rats and in man (10, 11, 20), indicate that the use of IPA-131 should not result in a radiation dose superior to that determined in common radioimmunotherapy (22, 23).

Finally, there seems to be a lack of correlation between the "modest" reduction in cell number seen in vitro compared to the dramatic effects seen in the orthotopic model. The demonstration of abundant lymphocytes, monocytes and macrophages at the site of previous tumour implantation of successfully treated animals clearly demonstrates that the immune system plays a major role in the clearance of the tumour cells. Thus, distinct mechanisms may have contributed to the dramatic anti-tumour effect specifically associated with the radiation treatment reported here. Certainly the beta irradiation of iodine-131 by itself after IPA-131 accumulation in tumour cells exerted an direct anti-neoplastic effect, which is what we see in the in vitro experiment. For the superior effects seen in the orthotopic model, a functional immune system seems to be necessary.

In conclusion, our approach provides measurable efficacy in a preclinical brain tumour model of a novel treatment for this devastating tumour type that does not share the toxicity of conventional cancer therapies. Furthermore, our result suggests that the new radio-iodinated amino acid may be a promising tracer for brain tumour therapy in humans. Hypothetically, radio-iodinated phenylalanine might turn out to be a very effective diagnostic and therapeutic tool for various types of tumours.

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References


