ORIGINAL ARTICLE

Taylor&Francis healthsciences

Biopolymer-Mediated Suramin Chemotherapy in the Treatment of Experimental Brain Tumours

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Acta Oncologica Vol. 43, No. 3, pp. 259–263, 2004

Suramin inhibits tumour growth and neoangiogenesis by blocking several growth factor receptors. In this study the toxicity and efficacy of intralesional delivery of suramin incorporated in a controlled-release polymer were assessed in a rat 9L tumour model. Initially, the toxicity of the compound was evaluated in adult Fisher 344 rats. The animals were intracerebrally implanted with an ethylene vinyl acetate copolymer. These experiments showed early toxicity in the rats implanted with a 50% load-polymer and 100% mortality within 48 h, whereas in rats implanted with a 33% load-polymer only transient behavioural changes were observed. In a second experiment the rats were stereotactically implanted with 9L cells in the frontal region. Two days after inoculation of cells, the animals were divided into two groups: one group received a 33% suramin load-biopolymer at the tumour implantation site, while the control group received polymer implants only. The interstitial release of suramin in the brain did not produce any improvement in survival of 9L tumour-bearing rats, with a mean survival of 14.2 ± 1 days for the suramin-treated group versus 13.8 ± 2 for the control group ($p = 0.82$). We conclude that intralesional polymer-mediated chemotherapy with suramin does not prolong survival in rats with intracerebral 9L tumours.

Received 17 July 2003
Accepted 13 November 2003

Despite the progress that has been made in surgery, chemo- and radiotherapy, malignant gliomas still have a poor prognosis (1). Malignant gliomas are characterized by autocrine stimulation based on secretion of growth factors such as EGF, b-FGF, PDGF and/or over expression of their constitutively activated receptors (2–4). Neoangiogenesis is indispensable for tumour growth and is mediated by endothelial proliferation induced by growth factors. Therefore attempts have been made to control tumour growth by inhibiting tumour-induced vasculogenesis (5, 6). Suramin, a polysulphonated naphthylurea synthesized in 1916, was originally used as an antiparasitic agent. In the past decade, it been demonstrated that suramin has an unexpected anti-tumour activity. This polyanionic compound can inhibit the activity of a variety of cytoplasmic and nuclear enzymes, and is able to block the receptor-binding process of b-FGF, EGF, PDGF, VEGF, TGF-α, IGF-II and IL-4, thus interrupting the autocrine loop underlying tumour growth (7–9). Suramin has demonstrated in vitro antiproliferative activity against a variety of human cancer cell lines, fresh human tumour specimens and antiangiogenetic activity (10–12). In clinical trials, suramin was effective against neoplasms such as lymphoma (13) and metastatic prostatic carcinoma (14). In experimental studies the efficacy of suramin in blocking the growth of human and rat glioma cell lines in vitro has been demonstrated (15, 16), but the efficacy of suramin in the treatment of gliomas in vivo is subject to debate. Olson et al. (15) found no improvement in survival of rats bearing 9L brain tumours when treated with a 7-day cycle of intraperitoneal (i.p.) suramin 7 mg/kg/day, and a reduced survival time when treated with a 35 mg/kg dose i.p. Takano et al. (16) reported a dose-dependent reduction of BudR-labelling but no significant reduction in tumour volume by using the C6 brain tumour model treated with suramin, 10–60 mg/kg i.p., given on alternate days. A reduced survival time was observed with 200 mg/kg suramin. However, Bernsen et al. (17) reported inhibition of tumour growth and reorganization of vascular architecture of subcutaneous human glioma xenograft in nude mice after treatment with 20 mg/kg suramin given 3 times a week, for 6 weeks. These discrepancies in results could be related to the poor penetration of suramin through the blood-brain barrier (BBB) (18) and the relevant systemic side effects of suramin at the dosages required to reach therapeutic

 ISSN 0284-186X
DOI: 10.1080/02841860310023129

concentrations intracerebrally. Biopolymer-mediated drug delivery to the tumour bed bypasses the BBB and reaches a high local drug concentration while minimizing systemic toxicity. Suramin, a polyanionic, highly hydrophilic structure with high molecular weight, is not metabolized in the brain. All these factors contribute towards prolonging the permanence of intralesionally administered suramin in the brain. In this study, an ethylene vinyl acetate copolymer (EVAc) was used to develop a biocompatible polymer (19) for direct intracerebral delivery of suramin, and subsequently tested in vitro and in the rat 9L glioma model.

MATERIAL AND METHODS

Experiment design

In vitro. For in vitro experiments, EVAc-suramin biopolymers were tested against 9L cell culture to confirm that suramin was not inactivated by the intrusion procedure.

9L glioma cells (55) were grown at 37°C, 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. Before the experimental procedures, cells in exponential growth were harvested by EDTA/trypsin for 5 min at 37°C. The trypsinization was stopped by medium with 10% fetal calf serum (FCS) and the cells were centrifuged. A trypan blue exclusion test was performed. Cell count was done in triplicate. For the intracerebral implantation, the pellets were then resuspended in medium without any supplement, at a concentration of 10^7cells/ml.

Growth inhibition assay

9L glioma cells were plated in 12 multiwell culture plates (Nunc) at a concentration of 5×10^4 cells/well. Forty-eight hours after plating, the medium was replaced with conditioned medium, as follows. Two bars of EVAc-suramin (33% and 50% loaded) and a control bar of EVAc polymer empty were left in 4 ml RPMI 1640 medium supplemented with 10% (FCS) for 48 h. The three resultant conditioned media were added to the cells. Cells were evaluated daily with a phase contrast microscope for 5 days and were counted on the fifth day. This experiment was repeated twice in quadruplicate wells for each polymer.

Polymer preparation

EVAc (Elvax 40P; 40% vinyl acetate by weight) was obtained from the Du Pont Company, Wilmington, Del. Suramin (lot no. 28H1267) was obtained from the Sigma-Aldrich Chemical Company and was incorporated into the EVAc matrix using a modification of the procedure described by Rhine et al. (20). The carrier EVAc was extensively prewashed in absolute EtOH for 60 days, with daily EtOH change, than dried over a period of 2 weeks. The polymers were dissolved in methylene chloride (EVAc: methylene 1:9 w/v) and mixed with suramin 1:1 and 1:2 weight ratio by vortexing. After mixing, the suramin was quickly frozen in liquid nitrogen. A slow, drying procedure was performed, first at -70° for 24 h, then at -20° for 6 days, then at room temperature. This procedure yielded dry polymers loaded with 50% and 33% suramin, respectively. The resultant polymers were cut into triangle-shaped fragments. The weights were 11.7 mg $\pm$ 0.3 mg for the 50% and 8.8 mg $\pm$ 0.3 mg for the 33% suramin. Thus the average suramin dose implanted was 6 mg or 3 mg per animal. Polymers 100% EVAc without drug were obtained following the same procedure as that for controls. The polymers were sterilized for 1 h before experimental procedures in EtOH 70% for 10 min and than washed three times in sterile phosphate buffered saline (PBS) for 2 min each in order to remove residual EtOH.

In vivo. Adult male and female Fisher 344 rats with a mean weight of 260 g (220–300 g) were used for in vivo tests. The animals were managed in accordance with the Rules and Regulations of the Central Animal Laboratory of the Hannover School of Medicine. The rats were anaesthetized with 80 mg/kg body weight Ketamin Hcl 10% and 2 mg/kg body weight of Xylazin Hcl 2%, i.m.

The first experiment was designed to assess the toxicity of EVAc-suramin polymers after intracerebral implantation. Three groups of 8 rats each were used. Rats in group A received 50% EVAc polymer loaded with 6 mg suramin, rats in group B received 33% polymers loaded with 3 mg suramin, and the rats in control group C received a drug-free EVAc polymer. In a second experiment we implanted 9L cells in the rat brains, and the tumour cells were allowed to attach and to proliferate. Two days after cell inoculation, a biopolymer was inserted into the brain of the rats at the tumour implantation site. The animals were divided into groups of 10 animals each: the rats in group D received a 33% polymer loaded with 3 mg suramin. Control group E rats received drug-free EVAc implants.

Cells injection

The animals were mounted on a stereotactic head frame in a flat-skull position. After reflection of the periosteum, a burr hole was made with a 1-mm diamond drill in the calvaria on the right side, 2 mm lateral from the midline, 3 mm anterior to the coronal suture. A total of 5×10^4 9L cells were stereotactically injected at a depth of 3 mm, using a 25-ml Hamilton syringe. The hole was sealed with bone wax, the operative field washed with saline solution and the skin sutured.

Biopolymer implants

A 3×2 mm craniectomy was performed centred at the level of the implantation site. The dura was incised and a small corticotomy was performed. A bar of biopolymer was inserted at the corticotomy site. The operative field was covered with a fibrin sponge and the skin sutured.

Evaluation

The animals were examined daily for focal motor deficits, gait disturbance or behavioural changes such as passivity, fearfulness, decreased alertness or responses to contact. In the first experiment, the rats were killed after a 3-week observation period. In the second experiment we chose the appearance of severe and progressive neurological deficits, e.g. lethargy, gait ataxia, hemiparesis as endpoints of the experiment. Rats bearing 9L tumours usually die within 24 h of the appearance of these symptoms. The animals were killed with an anaesthetic overdose and fixed with in situ endovascular formalin perfusion. The scalp tissues were inspected for extracranial tumour masses. The brains were grossly examined for abscess, haemorrhage and hydrocephalus.

Histology

After sacrifice, the brains were removed and post-fixed in 10% buffered formalin for 24 h; tissues were then embedded in paraffin blocks, 5-µm-thick consecutive coronal slices were cut through the tumours at the level of the implant. Staining was done with haematoxylin-eosin according to standard techniques.

Statistical analysis

Statistical analysis for differences between groups was determined by analysis of variance (ANOVA); survival analysis was performed using Kaplan–Meier cumulative survival curves, validated by log-rank tests. Statistical significance was assigned to differences with $p < 0.05$.

RESULTS

In vitro

In the in vitro assay, progressive growth inhibition and detachment of the treated 9L cells was observed during the 5 days of suramin treatment. Daily inspection of the cells revealed the presence of suffering, detaching and floating cells. After 5 days, more than 90% of the cells had died.

In vivo

In the first experiment we observed early toxicity in the group A animals, implanted with 50% suramin load-polymer. These animals showed markedly reduced responses to contact, diminished alertness, loss of appetite, and with 100% mortality within 48 h. In group B rats, implanted with 33% load-polymer, only transient behavioural changes were noticed. No late morbidity was observed throughout the whole observation period until sacrifice. The group C rates had no mortality or morbidity throughout the whole experiment. Based on these result, only the 33% suramin load-polymer rats were tested in the 9L glioma model. In the second experiment the animals tolerated the tumour implant well (Fig. 1). One rat in group D died a few hours post-polymer implantation and was not included in the mortality statistics because death seemed to be related to the surgical procedure. The mean survival of EVAc-suramin treated rats in group D was 14.2 ± 1 days versus 13.8 ± 2 days for control group E. Survival analysis showed no significant difference ($p = 0.821$) in survival between the two groups (Fig. 2).

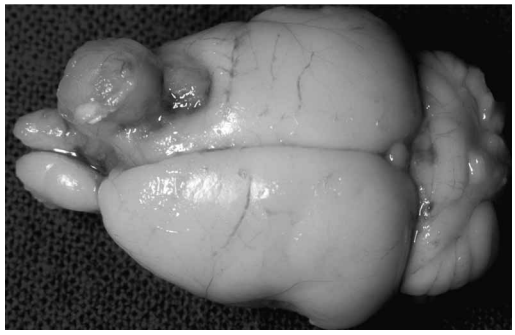

Fig. 1. Experimental 9L glioma in the right forebrain of the rat.

The histological analysis of the brains showed the presence of tumours in all animals in groups D and E. The control rats had typical 9L tumours with frequent mitosis and small necrotic areas. No haemorrhages could be seen. The suramin-treated animals had similar tumours. Again, intratumoral haemorrhages were absent. Necrotic areas could be detected, but these were no bigger or more numerous than those in the control animals. However, in contrast to the controls, we observed the presence of calcifications within the necrotic regions. No abscess or hydrocephalus was seen (Fig. 3).

DISCUSSION

The complete mechanism of action of suramin is unclear. Suramin binds strongly to a variety of plasma proteins and enzyme systems leading to multiple biological effects that could contribute to its various anti-tumour effects. Suramin shows inhibitory effects on growth factor binding to their receptors, as well as inhibition on protein kinase C, topoisomerase II, reverse transcriptase and iduronate

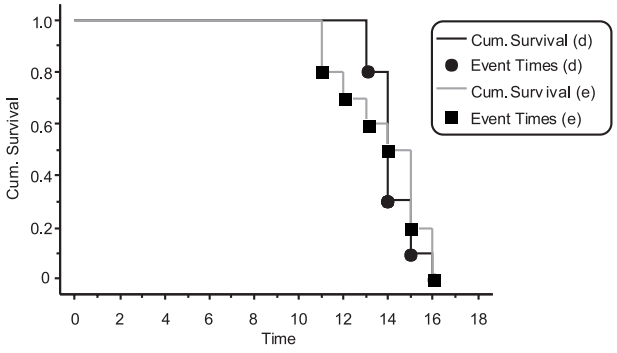

Fig. 2. Kaplan–Meier cum. survival plot for Group D and E log-rank test (Mantel-Cox) for this analysis: $p = 0.821$.

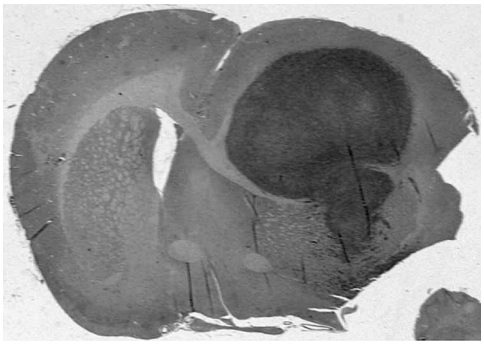

Fig. 3. Histological cross-section of the implanted glioma in the rat. Haemalum staining. Magnification $\times 40$.

sulphatase (21, 22). The inhibition of glycosaminoglycan catabolism causes the accumulation of heparan sulphate with angiostatic and antiproliferative effects (23). Suramin has been shown also to inhibit neovasculogenesis mediated by bFGF, VEGF, PDGF, EGF and other peptides. Among suramin side effects, adrenal insufficiency, leukopenia, thrombocytopenia, coagulopathy and polyneuropathy have to be reported (24–26). Our in vitro data suggest that the duration of exposure to the drug is important because of the slow onset of the drug's inhibitory growth mechanism.

In a subcutaneous glioma xenograft, Bernsen et al. (17) reported significant suppression of tumour growth after 4–5 weeks of treatment. One possible explanation for the effectiveness of suramin in that model is the absence of the BBB and the different period of observation of treatment. A rat bearing a brain 9L glioma usually dies within 15 days.

In the present study, suramin intruded in an EVAc matrix led to the in vitro inhibition of 9L glioma cell growth but did not show efficacy when seeded in 9L brain tumours. In our experimental model we were unable to find any improvement in survival after intralesional suramin therapy in rats with 9L brain tumours. We chose the rat 9L tumour model because of its 100% tumour take in Fisher 344 rats, its growth in a reproducible way in terms of shape and size and its tendency consistently to reach a volume that is incompatible with life. As 9L cells are sensitive to suramin in vitro, it is impracticable to explain the inability of suramin to act in vivo against experimental 9L tumours with an inherent resistance to the cell line. In this study the discrepancy between in vivo and in vitro results might be due to mechanisms of chemoresistance expressed by 9L tumours in vivo owing to the interaction with other cell types. What is more likely is that suramin does not reach therapeutic concentrations in brain tumours without causing significant morbidity in the surrounding parenchyma. Although in this study we could not provide evidence to show that suramin has a therapeutic effect at higher doses, our data with 50% suramin loaded polymers underline the high toxicity of the compound in the brain. Morbidity is indeed dose related. The elevation of circulating glycosaminogycans and the related anticoagulant properties of suramin have been advocated as the cause of cerebral toxicity of the drug (15, 16). However, direct neurotoxicity is a potentially serious dose-dependent side effect of suramin. In the clinical setting, suramin caused polyneuropathy that could progress to generalized flaccid paralysis with bulbar and ventilatory involvement requiring intubation and evidence of conduction blocks at multiple sites (24, 26). Induction of neuronal cell death was also observed in post-mitotic neurons in brain primary cultures and could be related to the accumulation of cell adhesion molecules to the cell surface (27). In experimental studies, suramin induced intracerebral formations of cytoplasmatic membranous inclusion bodies of both oligodendrocytes and neurons (16).

Direct neurotoxicity might explain the early morbidity observed in our study with the 6-mg suramin loaded biopolymer. However, the precise mechanisms for this are unclear. Further studies including immunohistochemical staining of different cell types and cellular components are needed.

Although this study did not show any improvement in survival times with biopolymer-mediated suramin treatment in experimental brain tumours, the results are encouraging for the development of suramin analogues along with a lower level of neurotoxicity that could open new perspectives for future investigational trials on brain tumours.

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