10.1053.paor.1999.0202 available online at http://www.idealibrary.com on IDEAL

ARTICLE

Antiproliferative Properties of the Lazaroids U-83836E and U-74389G on Glioma Cells In Vitro

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The 21-aminosteroids (lazaroids) are a new family of steroid compounds that inhibit lipid peroxidation reactions. They are novel antioxidant agents, which have been shown to have antiproliferative properties on cancer cells and also are thought to prevent free radical-mediated blood-brain barrier damage. In order to understand the effect of lazaroids on glioma, we tested U-83836E and U-74389G at doses ranging between 0.1–100 μM on primary cultures of glioblastoma multiforme from three patients, rat C6 glioma cell line, and 5th subculture established from one of the patients. The effects of both compounds on cell proliferation were determined using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. U-83836E in the primary cultures was found to have 50% inhibitory concentrations (IC_{50}) of 6.30, 6.75 and 6.50 μM, respectively. The IC_{50} value of U-74389G was calculated as 91 μM in only one of the patients. On C6 glioma cells, while the IC_{50} of U-83836E was 45 μM, U-74389G showed no cytotoxic effect. On the 5th subculture, U-83836E had an IC_{50} of 37.5 μM, but the cytotoxic effects of U-74389G was less than in that of the primary culture. In conclusion, these compounds were found to be more cytotoxic in primary culture than the cell lines and there were also differences between their members in the inhibition of cell survival. (Pathology Oncology Research Vol 5, No 3, 223–228, 1999)

Keywords: glioma, C6, MTT, 21-aminosteroids, U-83836E, U-74389G

Introduction

21-aminosteroids (lazaroids) are a family of non-glucocorticoid steroid compounds which have been developed as a lipid peroxidation inhibitor.[3,4] They have been shown to have a novel antioxidant capacity especially in neural tissue of animals exposed to trauma, subarachnoid haemorrhage, and ischemia.[15,16,27] Hence, lazaroids have been demonstrated to protect blood-brain barrier (BBB) in either in vitro or in vivo models of ischemia, and also subarachnoid hemorrhage.[12,14,27] Since the participation of free radicals in the mechanism of BBB damage and brain oedema is the proposed theory, some lazaroids have been used in the tumor implantation models to investigate their effects on brain oedema and also tumor volume, but controversial results have been reported.[2,18,21]

The inhibitory effects of 21-aminosteroids on cancer cells have been demonstrated in vitro.[17] Since there are structural similarities between lazaroids and glucocorticoids, the antiproliferative action of these compounds is attributed to their interaction with glucocorticoid receptors.[17]

It is known that glioma cells may change their morphology and some antigenic properties, depending on the degree of subculture; it is generally accepted that cultured cell lines become less differentiated as the time in vitro increases.[10] We, therefore, designed this study to gain an understanding of the effect of the lazaroids on a primary culture of glioblastoma multiforme and C6 glioma cell line, and also 5th subculture established from one of the patients. We chose two lazaroids; one of them, (–) 2-((4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl) methyl)-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopy-

Received: April 30, 1999; *accepted:* June 10, 1999

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 1219-4956/99/030223+06 $ 12.00/0

ran-6-ol dihydrochloride (U-83836E) is a novel antioxidant that inhibits lipid peroxidation,[15] as shown also in our previous study.[9] The other, 21-(4-(2,6-di-1-Pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl)-pregna-1,4,9(11)-triene-3,20-dione (z)-2-butenedioate (U-74389G) has been shown to reduce ischemic brain damage and also protect the normal brain from radiation injury.[5,24]

Materials and methods

Tumor specimens obtained from three patients at the time of surgery were examined histopathologically to confirm tumor diagnosis and a part of tumor tissues was prepared for cell culture as described previously.[19,30] After the tumor samples had been washed with phosphate buffered saline (PBS, without calcium and magnesium) in sterile petri-dishes and minced by scalpel, further dissociation of cells was achieved enzymatically, according to the method of Chen and Mealey.[7] Then cell aggregates, treated with 0.25% trypsin-EDTA solution and incubated 10–15 min at 37°C, cells were mechanically dispersed by using a 10 ml pipette. Trypsin activity was inhibited by adding growth medium (described below) and cells were centrifuged at 1000 rpm for 5 min at 4°C. Supernatant was removed and the pellet was seeded into 75 cm^2 flasks. Cells were cultivated in a 1:1 mixture of Dulbeccos modified Eagle's medium (DMEM) and F-12 supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution.[30] The cultures were humidified at 37°C and 5% CO_2 atmosphere and fed 2–3 times a week until they approached confluency (within 13–18 days).

Samples of monolayer culture grown on slide chambers (from ICN) were used for morphologic examination and GFAP staining by immonoperoxidase.[13] For drug experiments, monolayer cultures were washed with Hank's balanced salt solution (without calcium and magnesium) and treated with 0.25% trypsin-EDTA. After removal, the cells were centrifuged at 1000 rpm for 5 min at 4°C and counted with a Coulter counter. Cell viability was accessed by trypan blue dye exclusion, and found to be higher than 98%.

Drug preparation and MTT assay

Both lazaroids, U-83836E and U-74389G at 20 mM concentration were initially dissolved in dimethylsulfoxide (DMSO). They were then diluted to a 2.5 mM stock solution in fatty acid free bovine serum albumin in PBS (30 mg/ml). The highest concentration of both compounds (100 μM) was given by adding 10 μl of the stock solution to a 250 μl growth medium. Hence, the maximum concentration of DMSO was adjusted to 0.5% (the amount added to the culture medium together with the highest lazaroid concentrations) and was found to have no effect on cell viability when used alone. By using the stock solution, serial dilutions were made at a ratio of 1:10 in fatty acid free serum albumin to reach desired final concentrations of lazaroids ranging from 0.1 to 10 μM when added to 250 μl growth medium.[15]

It has been reported that the doubling time of short-term cultures of human glioblastoma may vary between 34–66 hours.[25] The duration of incubation, drug exposure and recovery period of primary cell cultures were selected and applied according to a protocol taken from the work of Thomas et al.[28] In brief, the cells at exponential growth phase and also at passage level 1 were seeded in 96-well microtiter plates ($3x10^4$ cells/ well), 10 wells for control (include only growth medium) and 10 wells for each tested lazaroid dose, and were incubated for 72 hours. The test compounds were freshly prepared and added to growth medium at concentrations of 0.1, 1, 10, 100 μM renewing the growth medium every day to achieve a total drug exposure of 72 hours. At the end of this procedure cells were washed gently with DMEM and allowed to recover for a total of 72 hours. Then drug cytotoxicity screening was performed using MTT colorimetric assay originally described by Mosman and modified by Alley et al.[1,22] MTT (from Sigma) was prepared freshly before each test as 5 mg/ml in PBS, and sterile filtered to remove undissolved particles. For each 250 μl growth medium, 25 μl MTT solution was added and incubated for 4 hours. After all growth medium had been removed, crystal particles of MTT were dissolved in 100 μl DMSO by shaking multiwell plates for 2–3 minutes. The absorbance of formazan dye was read at 550 nm using Uniskan II plate reader (Flow Laboratories, Herts, UK). All the tests were triplicated.

Since the formation of formazan as a product of MTT has been found to correlate with the number of living cells[6,22] and MTT assay was performed before density limitation of cell growth was occurred in control wells, the optical density read from the drug-treated wells was converted to a percentage of living cells against the control using the following formula; absorbance of treated cells in the each well x 100/the mean absorbance of control cells. The dose response curves were calculated for lazaroids at the above-mentioned concentrations and expressed as the mean percent fraction of control ± Standard Error of Mean (SEM). IC_{50} was determined by plotting the logarithm of the drug concentration against the mean percentage of living cells at each dose of lazaroids, using a software program.[23,29] Statistical significance was ascertained by one way analysis of variance, followed by Tukey's multiple comparison test.[23]

Rat C6 glioma cell line – Because of the high formazan density which is over the range of the plate reader, we were unable to determine the cytotoxicity of lazaroids on C6 astrocytoma cell in the same duration of experiment

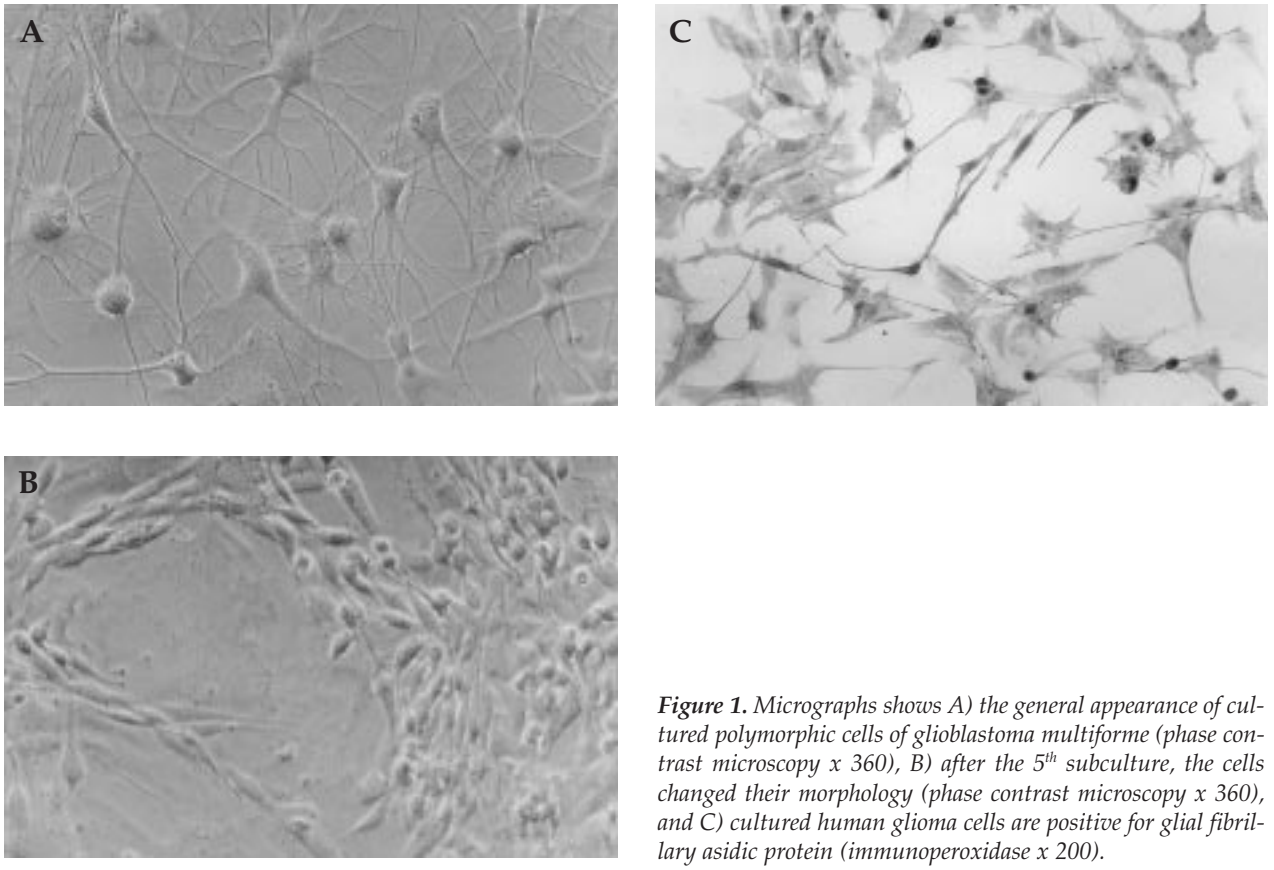


Figure 1. *Micrographs shows A) the general appearance of cultured polymorphic cells of glioblastoma multiforme (phase contrast microscopy x 360), B) after the 5th subculture, the cells changed their morphology (phase contrast microscopy x 360), and C) cultured human glioma cells are positive for glial fibrillary asidic protein (immunoperoxidase x 200).*

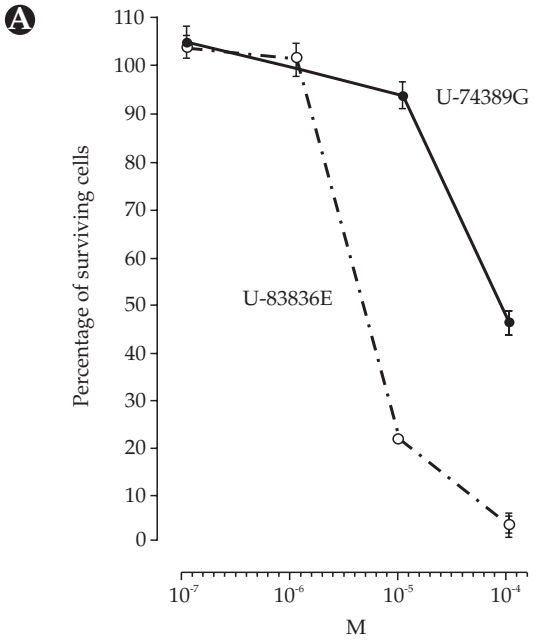
applied to human glioblastoma cell culture. Therefore, we first determined the doubling time of the C6 glioma cells in vitro, according to a method described previously.[25,31] In brief, cells were seeded in 24-well flat-bottomed culture plates (Costar) at a density of 10^4 cells/well. Throughout the experiment (6 days), quadruplet wells were trypsinized every 24 hours and living cells were counted using the trypan blue exclusion method. The plotted growth curve showed log phase starting at day one and reaching the plateau on day five. The number of cell divisions on the fifth day was determined by the following formula:[31] $n = ln (p/x)/ ln 2$, in which n = the number of cell divisions, p = the number of cells after n divisions, and x = the initial number of cells. Doubling time of C6 cells was assessed by dividing the duration of the experiment (5 d = 120 h) by the number of cell divisions. Since C6 cell doubling time was calculated as 20.77 h, MTT assay was performed at the end of 72 h period (24 h incubation, 24 h drug exposure and 24 h recovery period).

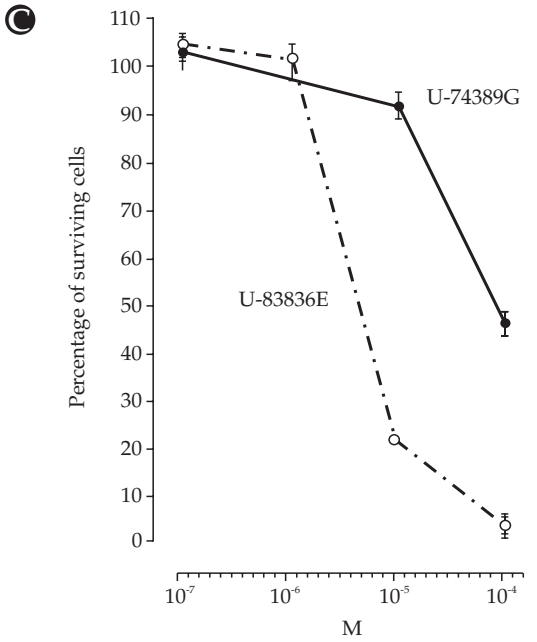
Results

Cultured cells of glioblastoma multiforme from three patients showed almost the same characteristics as described previously[26] *(Figure 1a).* The glioma cells derived from the one patient changed their initial morphology after the 5th subculturing (Patient 3, *Figure 1b).* The glial origin of the cells was demonstrated by immunoperoxidase staining with monoclonal antibody against GFAP, and found to be positive in more than 80% of cells *(Figure 1c).*

U-83836E was found to be more potent than U-74389G in reducing cell survival in all the experiments performed. U-83836E showed no effect on cell viability at the doses of 0.1 and 1 μM when compared to the control ($p>0.05$), but there was a significant difference between the consecutive doses after 1 μM in the reduction of living cell fractions ($p<0.05$). IC_{50} of U-83836E for the cells derived from the patients was calculated as 6.30, 6.75 and 6.50 μM respectively. When compared to the controls, there was no toxic effect of U-74389G on primary cultures at 0.1, 1 and 10 μM ($p>0.05$). However, there was a significant difference between 10 and 100 μM in reduction of cell survival ($p<0.05$). The effects of both lazaroids on primary culture are shown in *Figure 2*.

U-83836E reduced C6 cell proliferation in a dose-dependent manner, with the exception that there was no statistically significant difference between 0.1 and 1 μM ($p>0.05$). The IC_{50} value of U-83836E was found to be 45 μM, while U-74389G showed no effect on cell survival ($p>0.05$, *Figure 3a).* Since cytotoxic activity of lazaroids





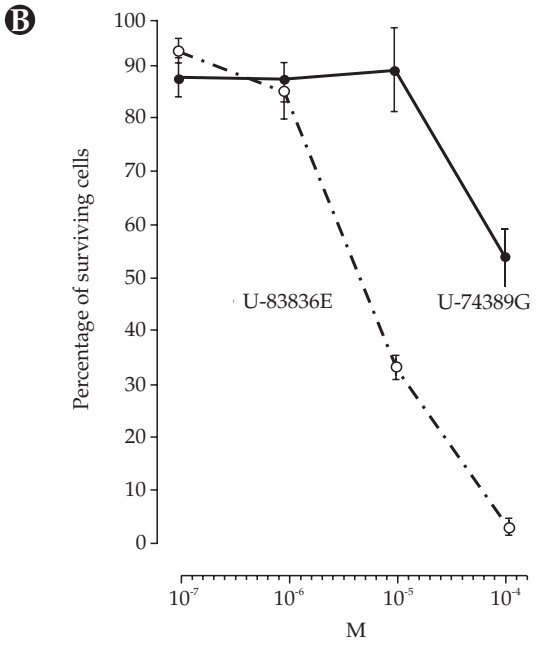


Figure 2. *The dose response curves show the percentage of living cells at indicated doses of both compounds against control ± standard error of mean. Antiproliferatve effects of U-83836E and U-74389G on promary cultures from the first patient (A) second (B) and third (C). IC_{50} values of U-83836E were 6.30, 6.75 μM, respectively. U-74389G had an IC_{50} of 91 μM only in the first patient (A).*

on C6 cells was found to be less than on primary cultures, we also planned to test these compounds on a cell line (5th subculture) established from one of the patients. At 0.1 μM, the reduction of cell survival brought about by U-83836E was not significant, but a significant difference was found between other consecutive doses of U-83836E ($p<0.05$). U-83836E had an IC_{50} of 37.5 μM and U74389G showed a small effect in reducing cell survival in this cell line *(Figure 3b).*

Discussion

This study demonstrates that U-83836E and U-74389G show cytotoxic properties on cultured glioma cells. At low doses they showed no significant effect on cell viability of the primary cultures but at higher doses their toxic effects became apparent. U-83836E was found to be more potent than U-74389G in decreasing the number of living cells. Since 21-aminosteroids are structurally similar to glucocorticoids, lacking only the C-11 and C-17 hydroxil groups which are responsible for glucocorticoid side-effects,[4] it is thought that they show their antiproliferative activities by interacting with glucocorticoid receptors.[17] This conclusion is drawn from several reports that glucocorticosteroids may inhibit the growth of neoplastic cells, including glioma.[8,32] Macuinas et al.[20] reported that dexamethasone caused cell death in a dose dependent manner in two glioblastoma cell lines derived from patients, but the IC_{50} of dexamethasone was found to be higher than tolerable by systemic delivery. On the other hand, in vitro studies have shown that the glucocorticoids are cytostatic, but not cytotoxic, at high cell densities in cultures of human glioma, besides enhancing cell survival and proliferation at low cell densities.[11] In present study it is not certain whether the reduction of cell survival by lazaroids is due to cytotoxic or cytostatic properties of the compounds on glioma cells, because the MTT assay does not distinguish these

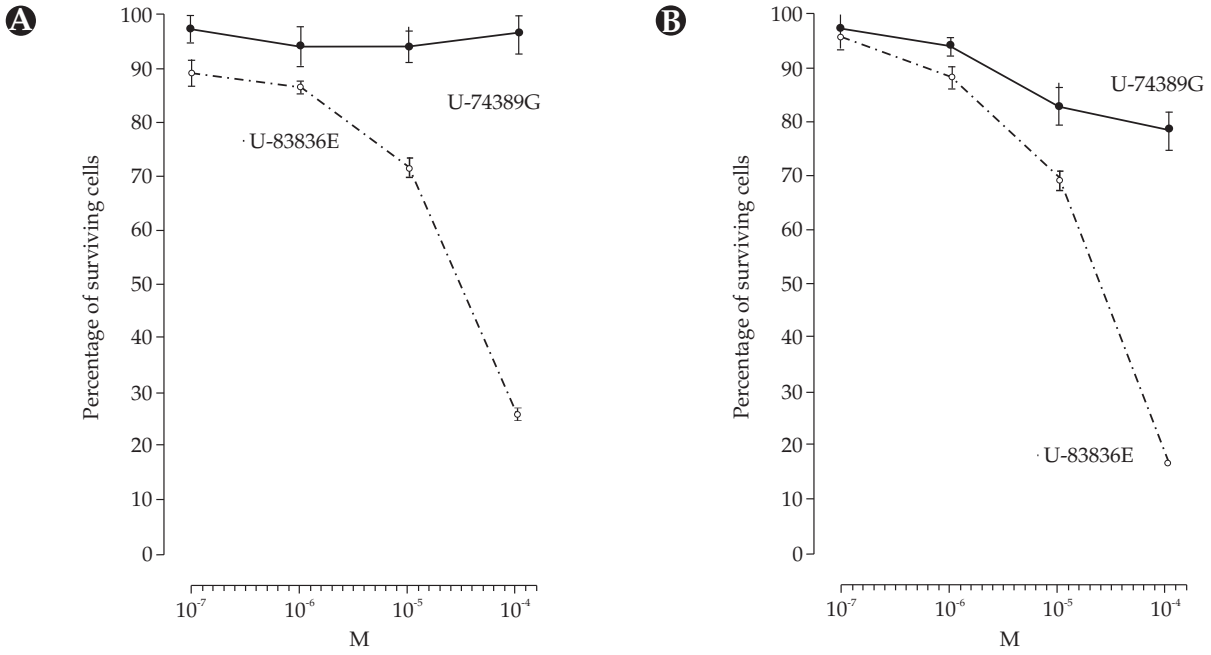


Figure 3. *The dose response curves show the percentage of living cells at indicated doses of both compounds against control ± standard error of mean. A) On the C6 cell line, IC_{50} value of U-83836E was 45 μM, but U-74389G had no effect on cell viability. B) On the 5th subculture, while U-83836E had an IC_{50} of 37.5 μM, U-74389G showed little antiproliferative actions on cells.*

two activities.[6] However, Kim et al.[17] have shown that the inhibitor activity of the lazaroid U-74500A varied depending on the drug exposure time and was a dose-dependent manner, suggesting that the lazaroid caused cell death due to cytotoxic action. In primary culture and C6 cell line, U-83836E caused reduction of cell survival in a dose-dependent manner for does over 1 μM, but the same effect was not obtained with U-74389G. These findings support the above-mentioned conclusion that inhibition of cell survival by lazaroids may be due to their cytotoxic effects on glioma cells.

While the cytotoxicity of U-83836E was lower on C6 glioma cells than on human primary cultures, U-74389G showed no cytotoxic effect at all on C6. A similar result was obtained in a previous study in which human breast cancer cells (MCF-7) were more responsive to lazaroids than mouse lymphoma cells (L5178Y), which suggested a varying sensitivity of different cell lines to lazaroids.[17] Interestingly, both compounds were found to be less effective in reducing the surviving cell fraction in the cell line established from a patients than they were in primary cultures but more effective than in the C6 glioma cell line in present study. Theoretically, another explanation for these results may be that glioma cells may partially lose their steroid receptors, depending on the degree of subculturing, although the steroid receptor expression was not a part of the present study. From the point of view of the oncolytic actions of lazaroids on cancer cells, a difference has been shown between their members both in this study and the previous one.[17] Which mechanism is responsible for such results is uncertain, but it seems that the more antioxidant capacity the agents have, the greater the effects that may be expected.

21-aminosteroids have been shown to attenuate the iron-induced vasogenic brain oedema in animals by protecting BBB.[15] In a rat model of cerebral carcinosarcoma (Walker 256), lazaroids (U-74006F and U-78517F) were incapable of decreasing the vascular permeability of the tumor, but capable of reducing neurological dysfunction by reducing tumor volumes.[18] In the present study, these compounds each showed cytotoxic actions on glioma cells depending on their inhibitory capacity in vitro. In conclusion, if they could be shown to reduce the tumor volume of glioma at tolerable doses by systemic delivery it is clear that they would be beneficial in the treatment of tumor-associated cerebral oedema, due to their antiproliferative effects on glioma cells.

References

1. *Alley MC, Scudiero DA, Monks A, et al:* Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 48:589-601, 1988.
2. *Arbit E, Rubinstein A, Di Resta G, et al:* The effect of the aminosteroids U-78517G on peritumoral brain oedema. Acta Neurochir (Wien) Suppl 51:158-159, 1990.
3. *Braughler JM, Pregenzer JF:* The 21-Aminosteroid inhibitors of lipid peroxidation: reactions with lipid peroxyl and phenoxy radicals. Free Rad Biol Med 7:125-130, 1989.

4. *Braughler JM, Hall ED, Jacobson EJ, et al:* The 21-aminostroids: Potent inhibitors of lipid peroxidation for the treatment of central nervous system trauma and ischemia. Drugs of Future 14:143-152, 1989.
5. *Buatti JM, Friedman WA, Theele DP, et al.* The lazaroid U-74389G protects normal brain from stereotactic radiosurgery-induced radiation injury. Int J Radiation Oncology Biol Phys 34:591-597, 1996.
6. *Carmichael J, DeGraff WG, Gazdar AF, et al:* Evaluation of tetrazolium-based semiautomated colorimetric assay: assesment of chemosensitivity testing. Cancer Res 47:936-942, 1987.
7. *Chen TT, Mealey J:* Microculture of human brain tumors. Cancer Chemother Rep 54: 9-14, 1970.
8. *Donalt TJ Jr, Higgins GM:* The effects of high levels of certain steriods on induced lymphocytic leukemia in the rat. Cancer Res 11:937-942, 1951.
9. *Durmaz R, Inal M, Angin K, et al:* The effects of MK-801 and U-83836E on post-ischemic reperfusion injury in rat brain. Acta Neurobiol Exp 59:99-104, 1999.
10. *Franks LM, Wilson PD:* Origin and ultrasutructure of cells in vitro. In: International review of cytology. (Eds: Bourne GH, Danielli FJ, Jeon KW), Academic Press, 1977, pp. 55-139.
11. *Freshney RI:* Effects of glucocorticoids on glioma cells in culture. Minireview on cancer research. Exp Cell Biol 52:286-292, 1984.
12. *Giese H, Mertsch K, Blasig IE:* Effect of MK-801 and U83836E on a porcine brain capillary endothelial cell barrier during hypoxia. Neuroscience Letters 191:169-172, 1995.
13. *Glick RP, Gettleman R, Patel K, et al:* Insulin and insulin-like growth factor I in brain tumors: Binding and in vitro effects. Neurosurgery 24:791-797, 1989.
14. *Hall ED, Andrus PK, Smith SL, et al:* Neuroprotective efficacy of microvasculary-localized versus brain-penetrating antioxidants. Acta Neurochir (suppl) 66:107-113, 1996.
15. *Hall ED, Braughler JM, Yonkers PA, et al:* U-78517F: A potent inhibitor of lipid peroxidation with activity in experimental brain injury and ischemia. J Pharmacol Exp Ther 258:688-694, 1991.
16. *Hall ED, Yonkers PA, McCall JM, et al:* Effects of the 21-aminosteroid U74006F on experimental head injury in mice. J Neurosurg 68:456-461, 1988.
17. *Kim RS, Zaborniak CL, Begleiter A, et al:* Antiproliferative properties of aminosteroid antioxidant on cultured cancer cells. Cancer Lett 64:61-66, 1992.
18. *King WA, Black KL, Ikezaki K, et al:* Novel 21-aminosteroids prevent tumor associated neurological dysfunction. Acta Neurochir (wien) Suppl 51:160-162,1990.
19. *Kornblith PL:* Role of tissue culture in prediction of malignancy. Clin Neurosurg 25:346-375, 1978.
20. *Maciunas RJ, Mericle RA, Sneed CL, et al:* Determination of the lethal dose of dexamethasone for early passage in vitro human glioblastoma cell cultures. Neurosurgery 33:485-488, 1993.
21. *Megyesi JF, Farrell CL, Del Maestro RF:* Investigation of inhibitor of lipid peroxidation U74006F on tumor growth and protein extravasation in the C6 astrocytoma spheroid implantation glioma model. J Neuro-Oncol 8:133-137, 1990.
22. *Mossmann T:* Rapid colorimetric assay of cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immun Method 65:55-63, 1983.
23. *Motulsky HJ, Stannard P, Neubig R, et al:* GraphPad Software, Inc, San Diego, 1994-1995.
24. *Müller TB, Haraldseth O, Jones RA, et al:* Perfusion and diffusion-weighted MR imaging for in vitro evaluation of treatment with U-74389G in rat stroke model. Stroke 26:1453-1458, 1995.
25. *Pertuiset B, Dougherty D, Cromeyer C, et al:* Stem cell studies of human malignant brain tumors. Part 2: proliferation kinetics of brain-tumor cells in vitro in early-passage cultures. J Neurosurg 63:426-432, 1985.
26. *Russell DS, Rubinstein LJ:* Pathology of tumours of the nervous system. Edward Arnold, London, 1989, pp 133-140.
27. *Smith SL, Scherch HM, Hall ED:* Protective effect of tirilazad mesylate and metabolite U-89678 against blood-brain barrier damage after subarachnoid hemorrrage and lipid peroxidative neuronal injury. J Neurosurg 84:229-233, 1996.
28. *Thomas DGT, Darling JL, Paul EA, et al:* Assay of anticancer drugs tissue culture: relationship of relapse free interval (RFI) and in vitro chemosensitivity in patients with malignant cerebral glioma. Br J Cancer 51:525-532, 1985.
29. *Tsuruo T, Iida H, Tsukagoshi S, et al:* Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 41:1967-1972, 1981.
30. *Unterman TG, Glick RP, Waites GT, et al:* Production of insulin-like growth factor-binding proteins by human central nervous system tumors. Cancer Res 51:3030-3036, 1991.
31. *Vincent AJPE, Esandi MC, Avezaat CJJ, et al:* Preclinical testing of recombinant adenoviral herpes simplex virus-thymidine kinase gene therapy for central nervous system malignancies. Neurosurgery 41:442-452, 1997.
32. *Wright RL, Shaumba B, Keller J:* The effect of glucocorticosteroids on growth and metabolism of experimental glial tumors. J Neurosurg 30:140-145, 1969.