Novel Ruthenium – Gamma-linolenic Acid Complex Inhibits C6 Rat Glioma Cell Proliferation *In Vitro* and in the Orthotopic C6 Model *In Vivo* After Osmotic Pump Infusion

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Abstract. *Aim: Gliomas are primary brain tumours. Gamma-linolenic acid (GLA) exerts anti-proliferative effects. Several ruthenium-containing complexes have antiproliferative effects and can be used as adjuvant therapies in cisplatin-resistant cancer. The present study reports on the anti-proliferative properties and effects on tumour morphology of a novel diruthenium-GLA complex (Ru_2GLA) and its comparison with GLA in the C6 rat glioma model both in vitro and in vivo. Materials and Methods: In vitro and in vivo experiments were performed on C6 glioma rat cells, and in an orthotopic model. Results: Ru_2GLA (100 μM) appears to be an inhibitor of C6 rat glioma cell proliferation. The nuclear area of Ru_2GLA-treated cells was 2.18-times larger than that of control cells, suggesting DNA replication occurred but mitosis was blocked in the G_2-M phase. Ru_2GLA (2 mM) inhibited C6 cell proliferation in vivo and the changes in tumor morphology confirm both cellular uptake and collagen fibre-binding in the extracellular matrix. Conclusion: Ru_2GLA appears to be a low-toxicity drug and a potential candidate for anti-proliferative therapy of glioma.*

Astrocytomas are the most common form of primary brain tumour and in the case of the most malignant astrocytoma, glioblastoma (GBM), the main treatment consist of surgical resection usually followed by radiotherapy and/or chemotherapy. Despite these often aggressive treatment options, the average survival of patients with GBM is between 9-12 months after surgical resection due to post-surgical recurrence of the tumor of almost 100% (1, 2). The main characteristics of GBMs are high rates of cell proliferation, areas of necrosis and accentuated angiogenesis (which is reflected in increased endothelial cell proliferation). These tumors can also migrate and invade the surrounding cerebral structures, primarily along myelinated fibre tracts and blood vessels.

Several new drugs are being developed for the treatment of glioma, including receptor tyrosine kinase inhibitors, inhibitors of key oncogenic signaling pathways, DNA-damaging drugs and anti-angiogenic drugs (3-7). The 18-carbon polyunsaturated fatty acid gamma-linolenic acid (GLA) has been reported to exert anti-tumour effects against several tumor types (8, 9). The inhibitory effects of GLA have been observed in gliomas, both in *in vitro* and *in vivo* animal studies and in human clinical studies (10-16). GLA is believed to act through multiple mechanisms including the production of reactive oxygen species, altered energy and lipid metabolism, cytochrome *c* liberation and subsequent caspase activation, followed by the induction of apoptosis (15, 17-20). In several studies the effects of GLA were found to be selective towards tumor cells, sparing normal astrocytes and improving the radiotherapeutic response of tumors (21, 22). Platinum-based drugs, such as cisplatin and carboplatin, have been successful in the treatment of certain types of solid tumours, although they are cytotoxic to the host and development of drug resistance occurs often (23, 24). Ruthenium-containing compounds have also been shown to exhibit anti-tumor activity with lower cytotoxicity than platinum-based drugs. These ruthenium-containing compounds can act by binding to both DNA and proteins, and inhibit RNA synthesis by DNA-dependent RNA polymerases (24). A recent study revealed that a ruthenium complex was toxic to C6 glioma cells *in vitro*, but not to primary astrocytes (25). The fact that ruthenium compounds

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are often cytotoxic to cisplatin-resistant cell lines makes them a potentially interesting novel treatment option (24).

The use of adjuvant therapies containing several active compounds led us to hypothesize about the possible efficacy of a single drug containing two active components, GLA and ruthenium, which could be degraded intracellularly. Paddlewheel-type structure dimeric ruthenium tetracarboxylates of general formula $[Ru_2(O_2CR)_4Cl]$ have been investigated for potential anti-inflammatory and antitumour properties (26-29). The present study reports the anti-tumor properties and effects on tumor morphology of the novel diruthenium-GLA complex $[Ru_2(aGLA)_4Cl]$, (Ru_2GLA), and a comparison with GLA alone in the C6 rat glioma model *in vitro* and in an orthotopic model in the Wistar rat *in vivo*.

Materials and Methods

Cell culture. C6 rat glioma cells were obtained from the ATCC (Manassas, VA, USA) and stocks were maintained frozen (liquid nitrogen) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and 20% glycerol. Stock cells were grown in DMEM containing 10% foetal calf serum and antibiotics (penicillin 50 U/ml, streptomycin 50 μg/ml). Cells in the exponential phase of growth were used, growing in 75 cm^2 flasks in a humidified atmosphere of 5% CO_2 with 95% air at 37˚C. For *in vitro* studies the C6 cells were grown on glass coverslips in 24-well plates. The cells were seeded at a density of $1\text{-}2\times10^4$ cells/well and the experiments were performed after 24-48 h. The cells were washed with 0.1 M Dulbecco's phosphate buffered saline-A, pH 7.2-7.4 (PBS) and used fresh or fixed with 4% formaldehyde in 0.1 M potassium phosphate buffer, pH 7.2-7.4 (KPB) for 30 min at 4˚C. In *in vitro* experiments, the effects of 150 μM GLA *versus* 100 μM Ru_2GLA- or Ru-alone were compared to control cells. These concentrations were chosen based on previous studies in tumor cells (17, 18, 20). Cell proliferation, cell viability and apoptosis were evaluated at 24-48 h by cell counting, trypan blue exclusion, lactate dehydrogenase release and TUNEL (Life Technologies, Carlsbad, CA, USA) labelling as previously described (17, 18, 20). Image analysis was performed using Sigma Scan (Systat Software, Chicago, IL, USA) and Image Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Surgical procedures. C6 rat glioma cells were grown in DMEM containing 10% foetal calf serum and antibiotics (penicillin/streptomycin). Cells in the exponential phase of growth were used and a suspension prepared in sterile saline at a concentration of 5×10^5 cells per 4-5 μl. Adult female Wistar rats of 250-350 g (n=6) were anaesthetised with an intramuscular injection of ketamine:xylazine, 10 mg:1.5 mg/100 g body weight to provide deep anaesthesia and analgesia. The rats were placed on a stereotaxic surgical table, a midline incision was made and a burrhole was drilled 0.48 mm anterior and 3 mm lateral to bregma. The C6 cell suspension was slowly injected into the striatum using a Hamilton syringe at a depth of 5.4 mm to the bone surface and the needle left *in situ* for 3 minutes before its removal. After 14 days Alzet osmotic pumps containing artificial cerebrospinal fluid (CSF), 5 mM GLA in artificial CSF or 2 mM Ru_2GLA were surgically-implanted and attached to Alzet brain infusion kits. These concentrations were chosen based on previous work and unpublished data from our laboratory (12). The pump infusion rate was 0.5 μl/h with duration of two weeks. After a further 14 days the rats were killed by transcardiac perfusion with 4% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. This procedure was approved by the Ethical Commission for Animal Experimentation of the Biomedical Institute (University of São Paulo) – protocol number 190/02.

Immunohistochemical (IHC) analysis by light microscopy. The perfused brains were cryoprotected in a solution of 20% sucrose in 0.1 M KPB overnight. The brain sections were cut on a freezing microtome Leica SM 2000R (Leica Microsystems, Wetzlar, Hessen, Germany), mounted and the sections were dried at 40˚C-50˚C for 2 h and were maintained at –20˚C until analysis. The slides were hydrated for 40 min and the endogenous peroxidase was blocked with a solution of 2% bovine serum albumin/2% pre-immune donkey serum/0.1 M PBS plus 0.2% triton X-100 (PBST). The sections were incubated at room temperature overnight with the respective primary antibody (1:100) diluted in PBST. Negative controls received only PBST. The slides were washed with PBST and incubated with the secondary antibodies (1:1000 in PBST) for 90 min. The slides were washed again with PBST and incubated with streptavidin (HRP) (1:100 in PBST) for 60 min. The reactions were developed with 0.04% 3,3'-diaminobenzidine (DAB) plus 0.03% H_2O_2. The DAB reactions were intensified with an OsO_4 solution (0.04%) for 30 min. All slides were counter-stained with 0.1% methyl-green, dehydrated and mounted with Permount (Fisher Scientific, Denver, CO, USA). The antibodies used in this study were against: collagen I (goat), collagen III (goat) and collagen IV (rabbit). All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Biotinylated secondary antibodies (antigoat and anti-rabbit) used for IHC were produced in donkey (Santa Cruz Biotechnology), and the streptavidin-biotin/HRP (horseradish peroxidase) was produced by GE Healthcare (GE, Buckinghamshire, UK).

Transmission electron microscopy procedure. After being post-fixed, tissues were dehydrated in alcohol, infiltrated and included in Epon 812 or LR White resin for semithin and ultrathin sectioning and observed by light microscopy and transmission electron microscopy (TEM). Semithin sections (3 μm) were stained with toluidine blue and ultrathin (80 nm) sections were contrasted with lead citrate and uranyl acetate and viewed at 80 kV on a JEOL transmission electron microscope JEM 1010 (JEOL, Tokio, Japan).

Synthesis $[Ru_2(aGLA)_4Cl]$. Synthesis and characterization of the compound $[Ru_2(aGLA)_4Cl]$, where aGLA is an anion derived from GLA was described by Ribeiro *et al*. (29). The $[Ru_2(aGLA)_4Cl]$ used in this study was synthesized and characterized at the Chemistry Institute of the University of São Paulo (Figure 1A).

Statistical analysis. All data are presented as mean±SEM. Statistical differences were determined by one-way ANOVA with *post-hoc* Tukey's test and $p<0.05$ was considered significant.

Results

In vitro studies of the effects of Ru_2GLA. In vitro studies found Ru_2GLA to be a potent inhibitor of C6 rat glioma cell

proliferation *versus* ruthenium-alone (Ru) *i.e.* $[Ru_2(O_2CCH_3)_4Cl]$, GLA-alone, and GLA-plus-Ru at concentrations of 150 μM and 100 μM respectively (Figure 1B). Of particular importance is the finding that GLA-plus-Ru present in the culture medium, did not exert the same inhibitory effects on proliferation as the Ru_2GLA complex. In these cells 150 μM GLA alone had a short-term inhibitory effect, with a slight apoptotic effect which was lost after 48 h (Figure 1D) (present data and 20). The effects of Ru_2GLA were not apparently caused by altered cell viability or necrosis since viability remained at approximately 97-99% in all cases and lactate dehydrogenase (LDH) leakage to the culture medium did not increase above 5% of total activity with treatment. C6 LDH activity: control 42.8±7.1 (1.92%); GLA 57.9±8.7 (2.15%); Ru_2GLA 80.7±13.7 (3.62%), $p<0.05$ *vs*. control; C6 total LDH activity 2227±23.8 (100%); all activities are expressed in nmoles/min/mg cell protein, n=4. Apoptosis was not detected by TUNEL analysis suggesting the principal effect was through non-cytotoxic mechanisms. Analysis of cell morphology found that Ru_2GLA cells contained many intracellular inclusions which had the appearance of lipid droplets (Figure 1E-F). Image analysis found that the nuclear area of Ru_2GLA-treated cells (83.66±2.52 μm^2, n=450 cells) was 2.18-times larger than control cells (38.35±0.802 μm^2, n=450 cells) ($p<0.001$) suggesting DNA replication occurred but mitosis was subsequently blocked (G_2-M blockade). No change in nuclear area was found for GLA (data not shown).

In vivo studies of Ru_2GLA effects. Representative images of semi-thin sections stained with toluidine blue are presented in Figure 2. In the control, tumor areas of intense proliferation can be seen with many blood vessels present in the tumor tissue. Treatment with 5 mM GLA reduced the number of proliferating cells and areas of necrosis and apoptotic cells, were visible throughout the tumor. The presence of 2 mM Ru_2GLA caused a marked change in tumor morphology, with the appearance of many large cells apparently filled with lipid droplets.

These findings were similar to the morphological changes seen in the *in vitro* experiments presented in Figure 1. Immunohistochemical labelling of extracellular matrix components in C6 tumours *in vivo* provide evidence that collagen types I, III and IV are expressed in this glioma model (Figure 2). These experiments support the description of collagen fibres in the study presented in Figures 3-5. In the control tumor, C6 cells have morphological features typical of highly proliferative and synthetic cells, containing many small cells with a low cytoplasmic/high nuclear area ratio and well-developed rough endoplasmic reticulum (Figures 2 and 3).

Common features in the tumor were migrating cells with filopodia clearly visible in many cells (Figure 3A-C). Extracellular matrix (ECM) was abundant in the C6 GBM model and both fibrillary and non-fibrillary matrix was found close to the tumor cells and blood vessels (Figure 3A, C and D). This ECM was not seen in the normal brain adjacent to the tumour. As shown in Figure 2, at least three collagen types were present in this tumor ECM, collagen I, III, and IV. The C6 GBM model is a highly angiogenic tumour (Figure 2) and angiogenesis is important not only for nutrition but also for tumour cell migration and invasion along the intra-tumoral blood vessels (30, 31). We frequently observed tumour cells attached to the basement membrane of tumour blood vessels, apparently using the basement membrane as a migratory substrate (Figure 3A and B).

Interestingly, this intense angiogenesis did not impede the observation of foci of necrosis in several areas of the tumor mass. The infusion of 5 mM GLA caused marked tumour necrosis and apoptosis was also frequently seen (Figure 4) in comparison to control tumor (Figure 3). Indeed, after GLA treatment, it was rare to find normal C6 cells as the majority showed signs of damage.

There was a visible decrease in cell number per unit tumor area, and pyknotic nuclei, membrane blebbing and organelle damage was often seen (Figure 4C, D and F). The ECM did not appear to be as abundant as that observed in the control group and the ECM was more non-fibrillar (Figure 4A-D). Many of the small capillaries seen in the GLA-treated tumor showed structural disorder, with gaps in the endothelial membrane and often lacked typical junctional complexes (Figure 4A, B and D). Occasionally, red blood cells were found in the tumor ECM, suggesting breakdown of the blood-brain barrier. Areas of necrosis were more abundant in the GLA-treated tumour, which may be related to altered vessel structure (Figure 4C, E and F).

Infusion of 2 mM Ru_2GLA was found to be well-tolerated as the animals showed no visible signs of serious side-effects related to direct infusion of the compound into the brain, and the tumour mass was visibly reduced *versus* control tumours, although insufficient data were available for statistical analysis due to the small quantitiy of Ru_2GLA available for preliminary testing *in vivo*. While the Ru_2GLA -treated tumour contained few apoptotic cells, normal tumour cells were difficult to find (Figure 5A-D). Most of the cells contained intracellular lipid droplets in abundance, with an increase in cytoplasmic area and with a greater number of filopodia present (Figure 5A and B). Electron dense material was seen deposited on collagen fibres, and is likely to be ruthenium (Figure 5D and E). Several cells were seen to take up electron-dense material by endocytosis (Figure 5F). Blood vessels were identified with gaps in the endothelial layer and the number of blood vessels was visibly reduced *versus* the control group. However, necrosis was uncommon in this experimental group.

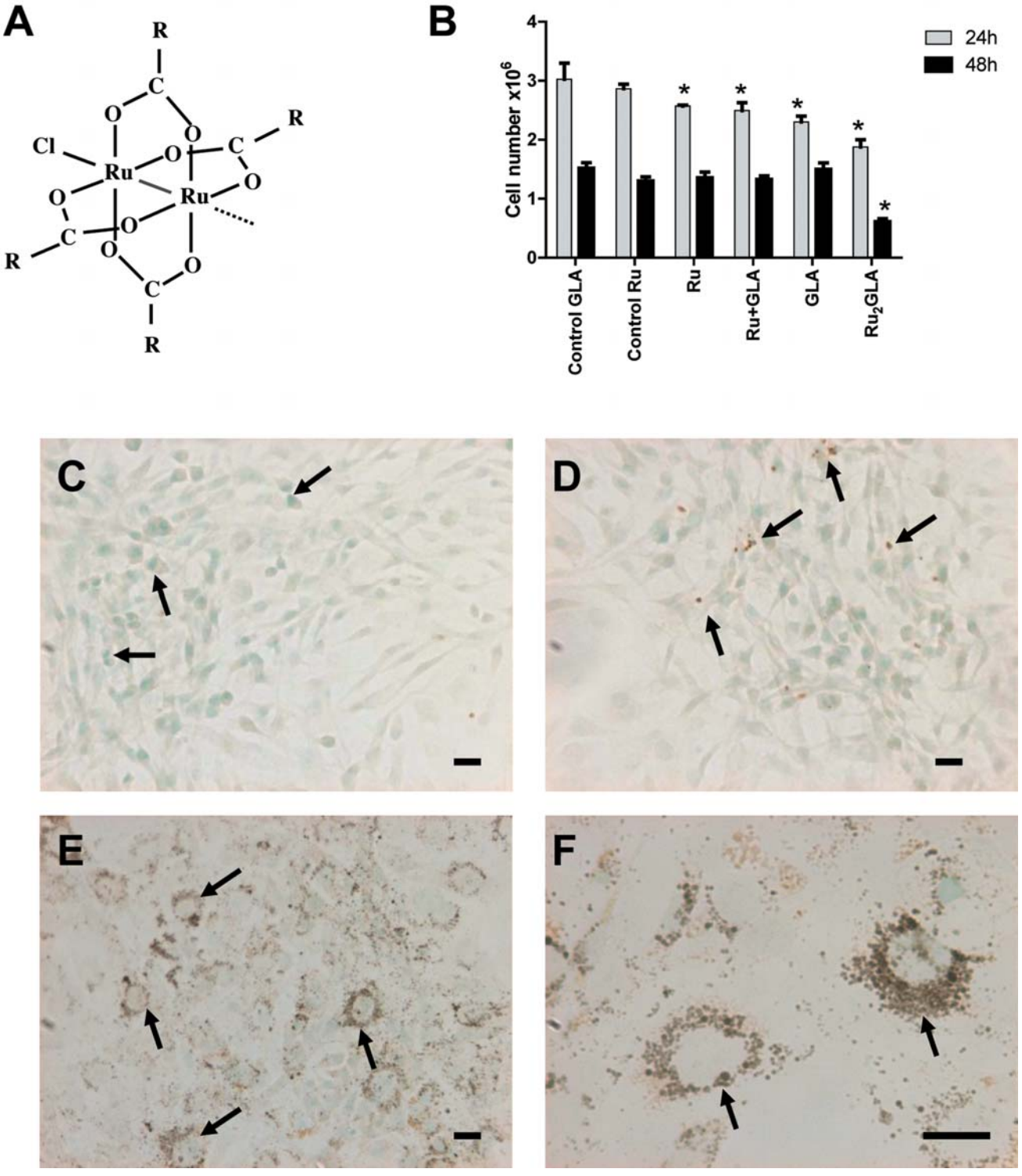

Figure 1. *A: Proposed structure of the Ru_2GLA complex. B: Effects of ruthenium and GLA on C6 glioma cell proliferation in vitro. Ru-ruthenium 100 μM; GLA 150 μM; Ctl Ru: vehicle control, ruthenium; Ctl GLA: vehicle control, GLA; Ru+GLA: ruthenium plus GLA 100 μM+150 μM, not in complex form; Ru_2GLA: ruthenium-GLA complex 100 μM. *p<0.01. Representative images of control, GLA and Ru_2GLA C6 cells after TUNEL labelling. C: Note control C6 cells with many mitotic figures marked by arrows. D: C6 cells after GLA treatment – note cells in apoptosis with small, brown nuclei marked with arrows. E, F: C6 cells after Ru_2GLA treatment – note cells filled with probable lipid droplets (E,F) and with larger nuclei than the control group (marked with arrows). Scale bar – 15 μm, C-E; - 10 μm, F.*

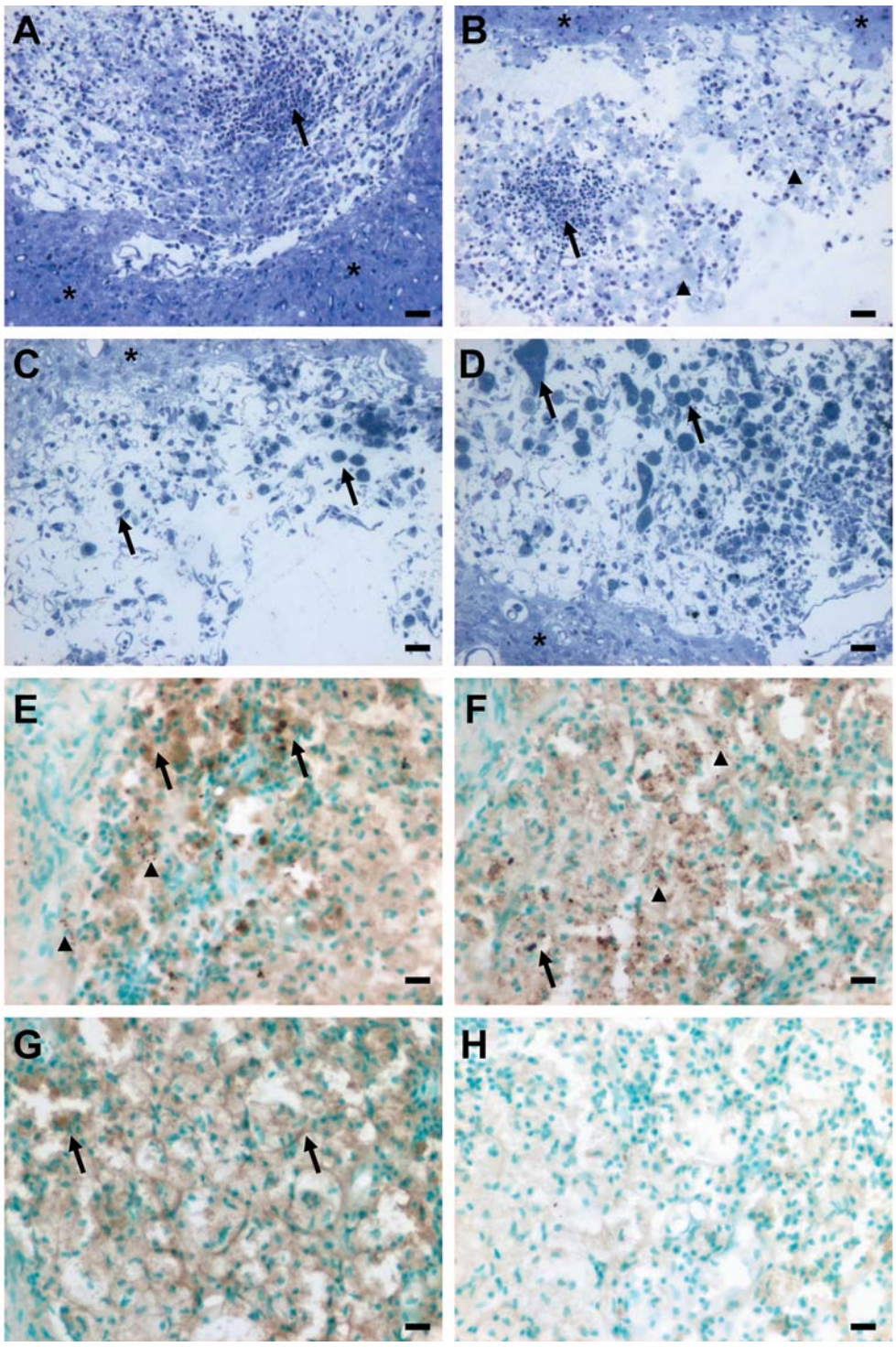

Figure 2. *Morphology of C6 glioma in vivo semi-thin sections (3 μm) of C6 tumour after 5 mM (GLA) or 2 mM (Ru_2GLA) infusion by osmotic pump into the tumor bed versus control tumour. Sections were stained with toluidine blue. Normal brain tissue is labelled with an asterisk (*) in A-D. Areas of cell proliferation are labelled with arrows in control (A) and GLA-treated tumour (B). Note areas of necrosis in GLA-treated tumor (B) marked with a triangle. In Ru_2GLA-treated tumor (C, D) note many cells filled with probable lipid droplets, marked with arrows. Scale bar – 30 μm. E-H: Immunohistochemical labelling of collagen type I, III and IV in C6 glioma tumour in vivo. This figure demonstrates three collagen types present in the tumor tissue. Collagen I (E) and collagen III (F) are present closely associated with cells, marked with arrows, and also distributed as small points in the extracellular matrix (ECM), marked with triangles. Collagen IV (G) is occasionally seen closely associated with cells and forms a net-like structure in the ECM. H: Negative control for collagen reactions. Scale bar – 15 μm.*

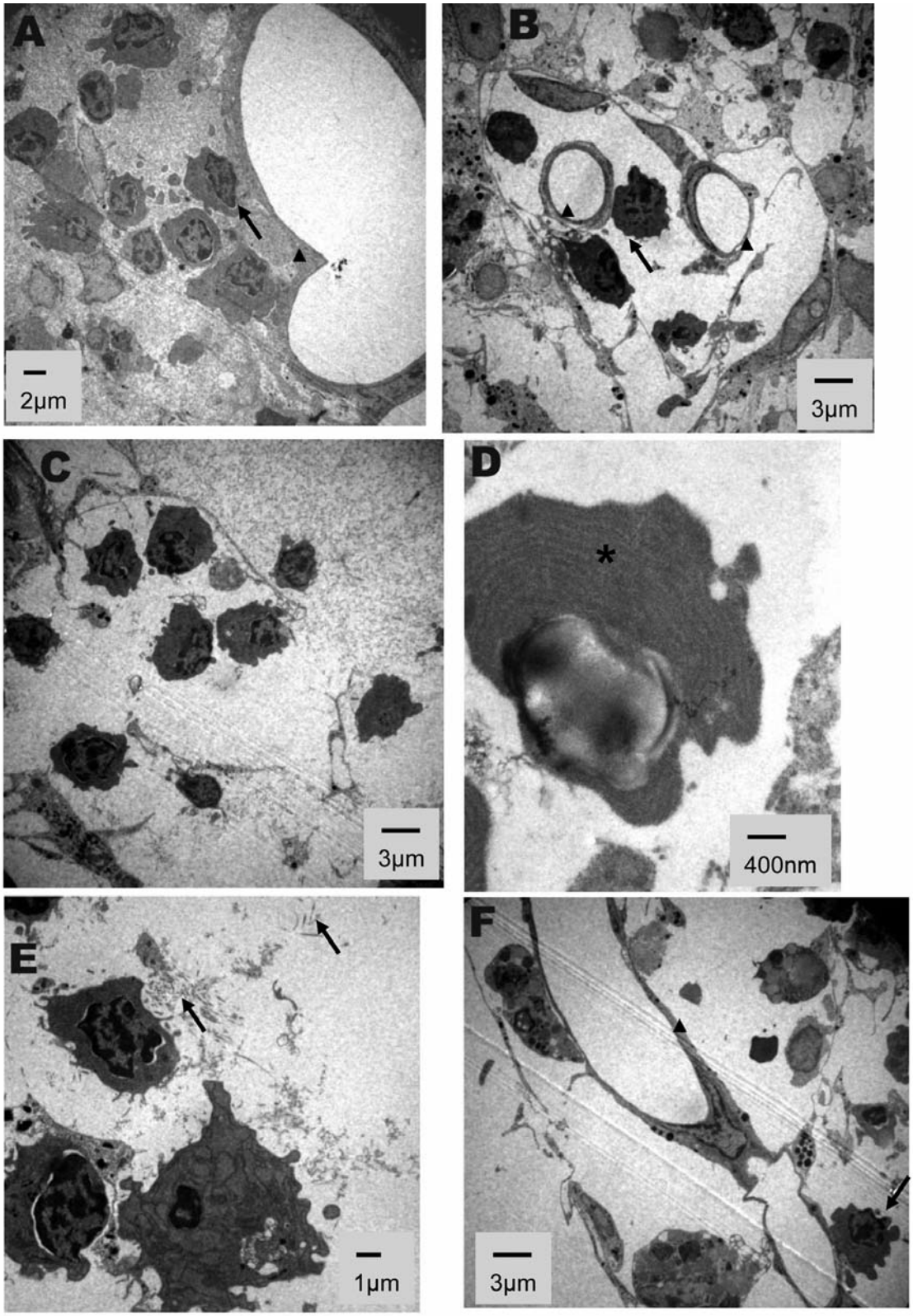

Figure 3. *C6 glioma model in vivo. A, B, F: C6 tumor cells close to blood vessels with well-preserved structure (marked with triangles) and tumor cells on basal membrane (marked with arrows). C, E: Tumor mass showing abundant extracellular matrix, note the presence of fibrillary (marked with arrows) and non-fibrillary matrix. D: Tumor cell with abundant rough endoplasmic reticulum, marked with an asterisk (*).*

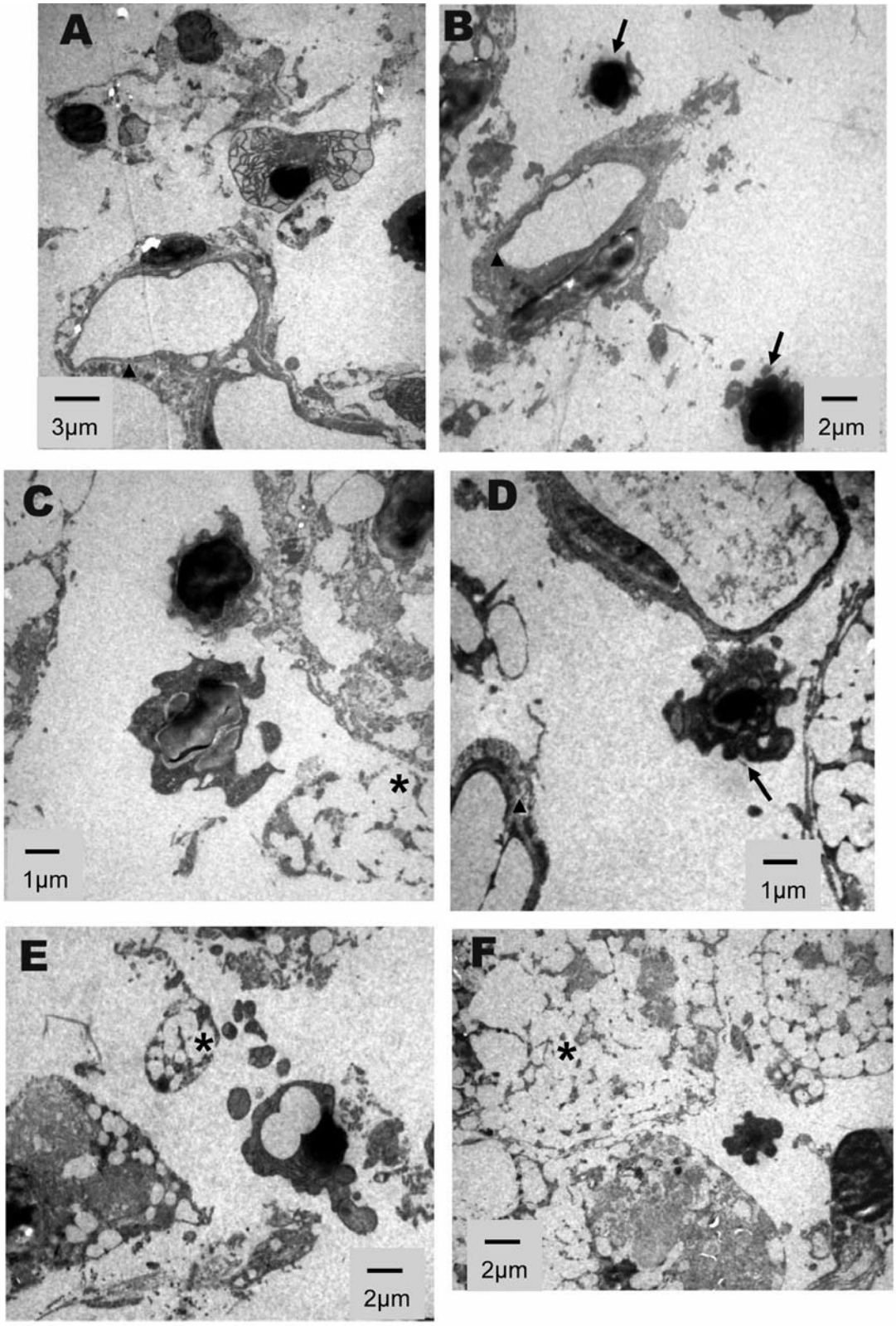

Figure 4. *C6 glioma after 5 mM (GLA) infusion by osmotic pump into the tumour bed. A, B: General view of the tumor after GLA treatment, showing altered tumor cell and vessel morphology (marked with triangle). B, C, D: Note tumor cells in apoptosis with pyknotic nuclei and membrane blebbing (marked with arrows). C, E, F: Note the intense necrotic process, marked with an asterisk (*).*

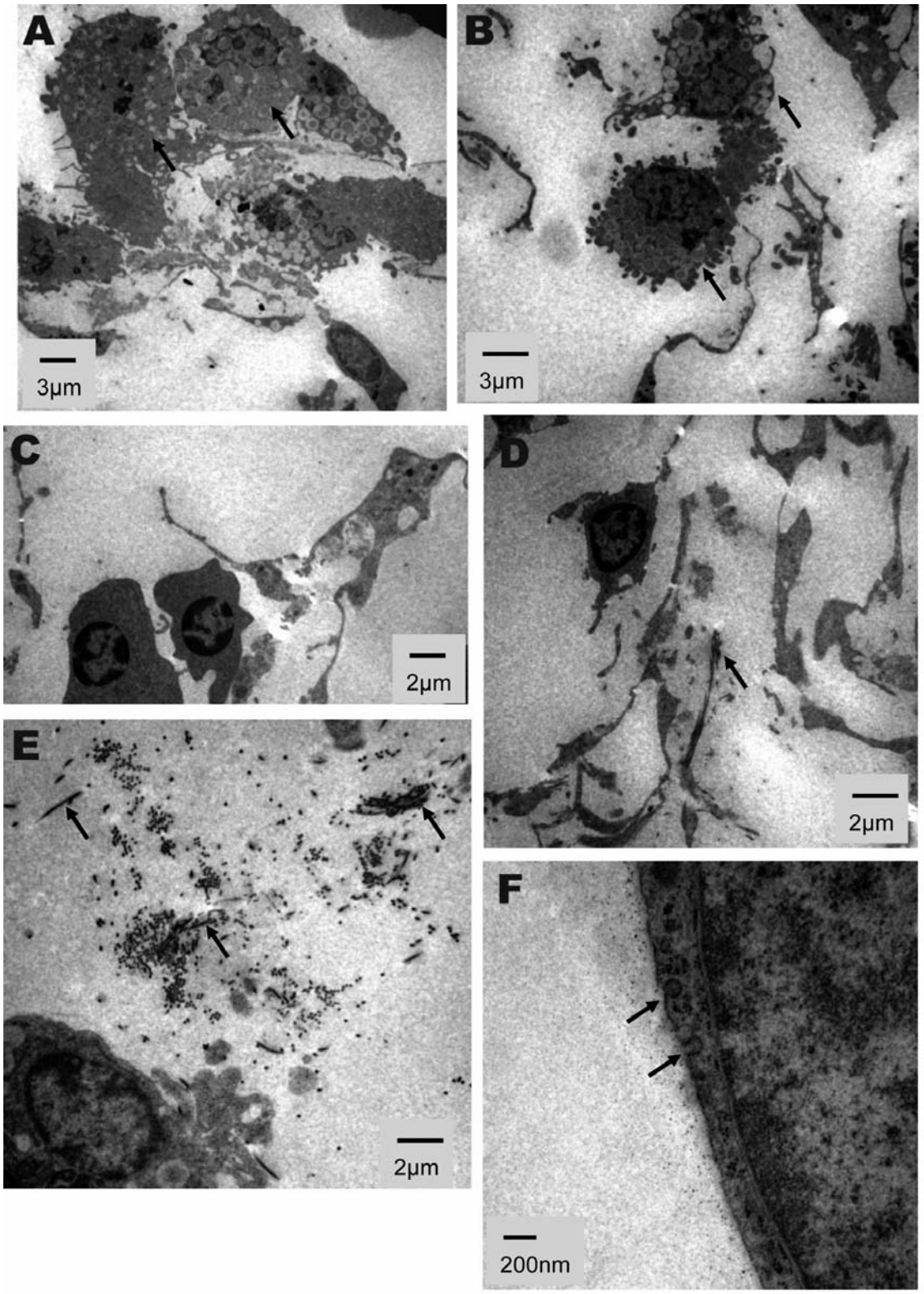

Figure 5. *C6 glioma after 2 mM (Ru_2GLA) infusion osmotic pump into the tumor bed. A, B: Note the presence of an altered cell type in the tumor mass which is large and completely filled with lipid droplets (marked with arrows). B, C, D: Tumor cells are scarce and incomplete blood vessels are present. D, E: Note the presence of ruthenium binding to collagen fibres (marked with arrows). F: Ruthenium at the plasma membrane surface and within endocytic vesicles (marked with arrows).*

Discussion

The present study reports on a novel drug which combines the anti-tumor properties of the two single-drugs, ruthenium and GLA. Similarly to previous light microscopy studies, GLA-treated C6 gliomas presented many apoptotic and necrotic cells (16). The induction of apoptosis may be caused by the multiple metabolic alterations known to occur in the presence of GLA. GLA alters cell proliferation, intracellular signalling, energy metabolism, and migratory, invasive and inflammatory processes (12, 15-20).

Ru_2GLA strongly inhibited C6 cell proliferation and the very marked changes in C6 cell morphology *in vitro* seen after Ru_2GLA treatment supports the conclusion that the compound is readily taken up by the cells (29). Whether the complex is degraded intracellularly or stored in lipid droplets, for example, remains to be determined.

Ruthenium is thought to accumulate inside tumor cells through the activity of the iron transporter protein transferrin (32, 33). The presence of metal impregnated endocytotic vesicles reported in the present study is suggestive of receptor-mediated binding and uptake of Ru_2GLA. During DNA synthesis, Ru and related compounds can bind to DNA, thereby causing DNA damage and inducing cell-cycle arrest for repair (24). The finding that the nuclear area in Ru_2GLA -treated cells was 2.18-times larger than control cells suggests that DNA replication occurred but mitosis was subsequently blocked (G_2-M blockade). This finding is an indicator of the possible damage done to DNA during the S phase of the cell cycle in the presence of Ru_2GLA (29).

The ruthenium anti-metastatic drug, NAMI-A, has been found to alter cell migration *in vitro* in matrigel assay suggesting it interferes with cell adhesion properties (34). Cells exposed to NAMI-A also assume a different shape due to the drug's interaction with cytoskeletal actin filaments, causing the extrusion of filopodia and of large lamellopodia which is believed to increase cell interaction with the substrate (35). Of note was the fact that the Ru_2GLA-treated cells had an increased number of filopodia and membrane protrusions, which may alter glioma cell interactions with the ECM in our experimental tumor model. NAMI-A is known to inhibit both matrix metalloproteinase-2 and -9 (MMP2 and MMP9) activity in a dose-dependent manner *in vitro* (36, 37). It also binds strongly to collagen fibres and it is thought that this together with MMP inhibition is an important factor in the inhibition of invasion and angiogenesis (36). NAMI-A has also been shown to inhibit cell proliferation and induce apoptosis in endothelial cell lines, suggesting that ruthenium compounds could alter the angiogenic process (38, 39). The fact that vessel integrity was altered in the Ru_2GLA-treated tumors is of particular interest and suggests that this compound may have anti-angiogenic properties which need to be further investigated. The fact that in our *in vivo* model Ru_2GLA was found to impregnate collagen fibres in the tumor ECM also suggests that this novel compound could have indirect anti-MMP activity in a manner similar to NAMI-A. It has been proposed that prerequisites for antimigratory/metastatic properties of ruthenium compounds would be low cytotoxicity and G_2-M blockade (34).

The lack of cytotoxicity of NAMI-A has been linked to its improved tolerability in comparison to other metal-based drugs such as cisplatin (40). It is, therefore, encouraging that the Ru_2GLA complex tested in the present study does not appear to act through cytotoxic mechanisms, as shown by unaltered cell viability, LDH leakage and apoptotic index. This suggests that Ru_2GLA will be well-tolerated in future studies and host toxicity may be low *in vivo*.

In conclusion, the present study on reports the effects of a novel ruthenium-based drug which combines the anti-tumor properties of the two single drugs ruthenium and GLA and apparently improves their individual effects. Further studies are underway to determine toxicity *in vivo* and to identify the mechanisms of action of this novel compound.

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