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Novel carbonic anhydrase IX-targeted therapy enhances the anti-tumour effects of cisplatin in small cell lung cancer

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Brief Summary:

Small cell lung cancer (SCLC) treatment has not significantly progressed for over 30 years, but targeted therapy now has the potential to improve patient outcome. This study found that response to cisplatin can be markedly enhanced through combination treatment with a novel, small molecule inhibitor of carbonic anhydrase IX (CA IX), and that this response is associated with increased apoptosis and necrosis. These results strongly support inhibiting CA IX in SCLC patients receiving chemotherapy.

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Conflicts of interest: Profs Williams and Supuran are authors of a patent (Carbonic Anhydrase Inhibitors, PCT/EP2011/052156).

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Abstract

Small cell lung cancer (SCLC) has an extremely poor prognosis and methods of improving chemotherapeutic intervention are much sought after. A promising approach lies in inhibiting the tumour-associated enzyme, carbonic anhydrase IX (CA IX), which supports tumour cell survival. The aim of this study was to assess the potential of CA IX inhibition using 4-(3'-(3'',5''-dimethylphenyl)ureido)phenyl sulfamate (S4), for the treatment of human SCLC alone and in combination with cisplatin chemotherapy. Treating SCLC cell lines (DMS 79 and COR-L24) with 100µM S4 reduced viability *in vitro* and enhanced cell death when combined with 7µM cisplatin, most prominently under hypoxic conditions (0.1% O₂). When either cell line was grown as a xenograft tumour in nude mice, intraperitoneal injection of 50mg/kg S4 alone and in combination with 3mg/kg cisplatin led to significantly reduced tumour growth. Combination therapy was superior to single agents and response was greatly accentuated when administering repeated doses of cisplatin in DMS 79 tumours. The mechanism of therapeutic response was investigated *in vitro*, where S4 treatment increased apoptosis under hypoxic conditions in both DMS 79 and COR-L24 cells. DMS 79 tumours receiving S4 *in vivo* also displayed increased apoptosis and necrosis. Combining S4 with cisplatin reduced both the area of hypoxia and CA IX-positive cells within tumours and increased necrosis, suggesting hypoxia-specific targeting. This study presents a novel, targeted approach to improving current SCLC therapy via inhibition of CA IX, which enhances apoptosis and significantly inhibits xenograft tumour growth when administered alone and in combination with cisplatin chemotherapy.

Introduction

Small cell lung cancer (SCLC) remains one of the most aggressive types of cancer, with 5-year survival rates below 5% ¹. This dire prognosis has remained relatively unchanged since the 1980s with patients still relying on broad-range chemotherapeutic agents, often cisplatin plus etoposide, in combination with radiotherapy. Although response to first-line therapy is extremely high, patients almost inevitably relapse with therapy-resistant disease ². A number of molecular-targeted therapies have progressed to clinical trials, yet none to date have shown any benefit, highlighting the necessity for developing targeted strategies for the treatment of SCLC ³. Such strategies must be capable of diminishing tumour mass without the extensive off-target toxicity displayed by chemotherapeutics.

SCLC tumours proliferate rapidly and outgrow their blood supply, leading to areas of pathologically low oxygen. Hypoxia is extremely common in SCLC tumours and has been linked with poor survival ⁴. The presence of hypoxia within tumours has long been associated with resistance to therapy^{5, 6} and is known to induce additional aggressive traits including epithelial to mesenchymal transition (EMT) and stem cell features ⁷⁻⁹. The characteristics exhibited by hypoxic tumour cells highlight the importance of targeting these cells in the treatment of SCLC ¹⁰.

Under hypoxic conditions, activation of the hypoxia-inducible factor (HIF) signalling pathway orchestrates the expression of hundreds of genes involved in adapting and surviving low oxygen conditions. Among HIF's targets is carbonic anhydrase IX (CA IX) which is one of 15 carbonic anhydrase isoforms; all of which have the primary role of catalysing the inter-conversion of carbon dioxide and water into bicarbonate and protons ¹¹. Subsequently, intracellular pH is kept relatively alkaline while the extracellular environment becomes more acidic, aiding in extracellular matrix degradation and metastasis ¹². CAs are found ubiquitously throughout the body; however, CA IX is highly tumour-specific and is only expressed in healthy tissue within the gastrointestinal tract ¹³. Expression of CA IX is found

in 90% of SCLC biopsies and corresponds closely with hypoxic regions, highlighting its eligibility as a target for inhibition in SCLC ¹⁴.

The relevance of using CA IX inhibition to treat SCLC was demonstrated in a study investigating modifications in gene expression following *CA9* knockdown in fibrosarcoma cells. This study revealed differential regulation of multiple genes associated with SCLC signalling ¹⁵. Of note was a reduction in the expression of S-phase associated kinase 2 (*Skp2*), which is upregulated in SCLC and associated with cisplatin resistance ¹⁶. This further supports the use of CA IX inhibition when treating SCLC, particularly when combined with cisplatin chemotherapy.

The aim of this study was to determine the potential of targeting CA IX using the novel small molecule inhibitor, S4, in combination with cisplatin for the treatment of SCLC. Our data show that CA IX inhibition highlights a new therapeutic option for the treatment of SCLC, particularly when combined with cisplatin. The treatment induced apoptosis and led to greatly increased necrosis within tumours, resulting in significant inhibition of tumour growth.

Materials and methods

Cell maintenance

DMS 79 cells were kindly donated by Dr. Pettengill (Dartmouth Medical School, Hanover, USA) having been established in 1980¹⁷. COR-L24 cells were bought from European Collection of Authenticated Cell Cultures (ECACC) and were established and characterized in 1985¹⁸. Both cell lines were authenticated by the DNA Sequencing Facility (University of Manchester) and showed greater than 90% similarity in short tandem repeats (STR) profile when compared with the American Type Culture Collection database (ATCC). Furthermore, DMS 79 cells have been reported to possess the *p53* and *RBI* gene mutations¹⁹ and c-myc amplification²⁰, characteristic of SCLC. COR-L24 and DMS 79 cell lines display classic neuroendocrine properties typical of SCLC, supporting the use of both these cell lines as representative of the disease²¹. Neither patient had received platinum therapy prior to cell lines being derived. SCLC cells grew in suspended aggregates and were cultured in RPMI medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (SeraLab, West Sussex, UK) at 37°C in a humidified atmosphere of 5% CO₂. When cultured under hypoxic conditions, cells were kept in 0.1% O₂ at 37°C in an H35 Hypoxystation (Don Whitley Scientific, UK). Cell culture media was regularly screened for the presence of mycoplasma by PCR.

Western blot analysis

Cells were lysed in 75µL lysis buffer (10mM Tris (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM EGTA, 50mM NaF, 1mM sodium orthovanadate, 1% Triton X-100 v/v, 0.5% Nonidet P-40 v/v, 2mM leupeptin, 0.15mM aprotinin, 1.46mM pepstatin, and 1mM phenylmethanesulfonyl fluoride). Protein concentrations were established by Bicinchoninic acid (BCA) assay. Protein (20µg/lane) were separated on pre-cast gels (BioRad) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Schwalbach, Germany) via dry transfer using a Trans-blot Turbo transfer system (BioRad, Herts, UK). After blocking in 5% milk (Marvel)-TBS-T (137mM NaCl, 2.7mM KCl, 4.3mM di-

sodiumhydrogenphosphate, 1.4mM potassium-di-hydrogenphosphate, 0.1% Tween20), the M75 antibody (gift from Prof. S. Pastorekova, Bratislava, Slovakia) was added to blots diluted 1 in 6 together with anti- β -actin antibody (Sigma-Aldrich, Dorset, UK) diluted 1 in 75 000 overnight. Blots were then exposed to secondary anti-mouse Fc-specific antibody (Sigma) diluted 1 in 2500 for 1 hour and reacted with ECL1 and 2 (BioRad). Protein bands were visualized using a Chemidoc MP imaging system (BioRad) and quantified using Image J software, normalizing CA IX expression to internal β -actin control bands.

Treating cells with S4 and cisplatin

SCLC cells were plated at 7.5×10^4 cells per mL RPMI medium in 24 well plates (1.5 mL DMS 79 cell suspension) or T25 flasks (5 mL of COR-L24 cell suspension) to provide the most favourable growth conditions. S4 and/or cisplatin were added to the RPMI medium to give final concentrations of 100 μ M S4 (diluted in DMSO) and 7 μ M cisplatin (diluted in H₂O). DMSO and H₂O were used as vehicle controls. After 48 hours incubation under normoxic or hypoxic conditions, viability was assessed. To quantify relative numbers of viable cells, 30 μ L of cells were taken from each well and added to 30 μ L CellTiter-Glo solution (Promega, WI, USA) in a 96 well plate. Following 10 minute incubation, luminescence was read using a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Calcein / Ethidium homodimer-1 cell viability assay

In cytospun cells we simultaneously determined the live and dead cells using the LIVE/DEAD[®] viability/cytotoxicity assay kit (Molecular Probes) according to the manufacturer's instructions provided.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

In cytospun cells and tumour sections apoptotic cells were identified using the DeadEnd[™] Fluorometric TUNEL System (Promega, Hampshire, UK) following manufacturer's instructions provided.

Animal Studies

CBA nude female mice that have been bred in house were used for *in vivo* studies. Small clumps and single cells were suspended in serum-free RPMI containing 50% Matrigel (Scientific Laboratory Supplies, Surrey, UK) at a final concentration of 5×10^7 cells per mL. Mice were anaesthetized and injected sub-cutaneously on the lower back with 100 μ L of the cell suspension. Tumours were measured and mice weighed 3 times a week throughout the study. Tumour volumes were calculated by multiplying the length, width and depth of tumours. All procedures involving animals were performed in accordance with the UK Home Office Animal (Scientific Procedures) Act, 1986, and approved by the local University of Manchester Ethical Review Committee (PPL 40/3212 and 70/7760 held by Prof. K. Williams).

A dose of 50mg/kg S4 was given on a '5 day on, 2 day off' regimen for up to 3 weeks via intraperitoneal injection to model weekday administration. Mice received S4, cisplatin, or the combination once tumours reached 250mm³. Tumours were harvested at 1000mm³. Two hours prior to being culled, mice received intraperitoneal injection of 60mg/kg pimonidazole (Chemicon International Inc. CA. USA) to indicate hypoxic regions.

Immunohistochemistry

For antigen retrieval, slides were incubated in citrate buffer (pH 6.0) at 95°C for 30 minutes. Sections were then blocked in peroxidase block (Dako) followed by serum block (PBS with 20% normal goat serum, 0.1% bovine serum albumin (BSA) and 0.02% triton (Sigma-Aldrich)). The following antibodies were then diluted in serum block and added to slides: M75 diluted 1 in 6 (a kind gift from Prof. S. Pastoreková, Slovak Academy of Sciences, Slovakia); hypoxyprobe diluted 1 in 25 (Chemicon International Inc, CA, USA); Ki67 clone MIB-1 diluted 1 in 50 (Dako) and IgG diluted 1 in 50 as a negative control (Dako). Slides were then incubated in anti-mouse secondary antibody diluted 1 in 75 before being exposed to chromagen substrate (1mL DAB substrate with 20 μ L chromagen (Dako)). Samples were

counterstained to highlight nuclei using Gills haematoxylin. For H&E staining, sections were exposed to Gills haematoxylin followed by eosin stain.

Microscopy and analysis

Images were acquired using a [20x/0.80 *Plan Apo*] objective using the 3D Histech Panoramic Flash II slide scanner and analysed in Panoramic Viewer. Staining was quantified using particle analysis, area measurement tools and HistoQuant analysis in Panoramic Viewer or Image J.

Statistical Analysis

Differences between experimental groups were tested using either one or two way ANOVA. *P* values less than 0.05 were considered statistically significant.

Results

CA IX is expressed in hypoxic SCLC cells and xenograft tumours

SCLC cells grow in suspended clumps under normoxic conditions (figure 1A). DMS 79 cells grow in small, loose irregular spheroidal-like bodies, whereas COR-L24 cells grow in larger, tighter aggregates. Strong expression of CA IX protein was detected within SCLC cells exposed to hypoxic conditions *in vitro*, supporting the use of CA IX inhibition as a valid approach in these cells. When exposed to hypoxia *in vitro*, CA IX expression was upregulated by 16 hours and was further upregulated after 24 hours (figure 1B). Side by side comparison of the two cell lines suggested that hypoxic upregulation of CA IX in COR-L24 cells was approximately 50% of that observed in DMS79 cells at 24 hours (Supplemental figure 1). Treatment with the CA IX inhibitor, S4, reduced expression of CA IX *in vitro* in both DMS 79 cells and COR-L24 cells (figure 1C).

S4 reduces SCLC viability and improves response to cisplatin

DMS 79 cells were sensitized to S4 by hypoxic conditions, although a reduction in DMS 79 cells treated with S4 under normoxic conditions was also evident (figure 2). COR-L24 cells showed reduced viability in response to S4 in both normoxic and hypoxic conditions. When treating with cisplatin, cell death was seen in DMS 79 cells regardless of oxygen concentrations. COR-L24 cells were much less sensitive to cisplatin treatment. Combining S4 with cisplatin dramatically reduced viability in both cell lines, particularly under hypoxic conditions in DMS 79 cells. Less than 5% of DMS 79 cells and 40% of COR-L24 cells remained viable when exposed to combination therapy under hypoxic conditions. This extensive cell death surpasses that seen when cells are exposed to single agent therapy.

S4 and cisplatin therapy induces apoptosis under hypoxic conditions *in vitro*

The contribution of apoptosis to the impact of S4 and cisplatin on cell viability was evaluated in both normoxic and hypoxic conditions. DMS 79 cells were kept in hypoxia for 60 hours (figure 3A). COR-L24 cells appeared to be less tolerant to oxygen-deprivation, restricting the

hypoxia incubation for these cells to 48 hours (figure 3B). S4 alone had little effect on apoptosis in normoxia, but caused marked apoptosis in hypoxia in both cell lines. Cisplatin induced significant apoptosis in both conditions in DMS 79. The absence of cisplatin-induced apoptotic COR-L24 cells, is most likely a reflection of the shorter incubation time as compared to DMS 79 cells. The addition of S4 to cisplatin resulted in enhanced levels of apoptosis under hypoxic conditions in DMS 79 and COR-L24 cells.

S4 enhances the anti-tumour effects of cisplatin in SCLC sub-cutaneous xenografts

Treating mice bearing DMS 79 xenograft tumours with S4 resulted in an equivalent reduction in tumour growth as seen with cisplatin therapy (figure 4A). The time for tumours to reach a relative tumour volume three times that at the start of therapy (RTV3) was increased 1.6 fold following S4 therapy and 2.1 fold after cisplatin compared with control (figure 4B). When S4 was combined with a single dose of cisplatin chemotherapy, DMS 79 tumours took 3.6 times longer than control tumours to reach RTV3. This combination therapy is well tolerated *in vivo* as evidenced by the fact that all mice within these studies remained in good health and showed a gradual increase in weight up to an average of 6% by the end of the experiment (data not shown).

S4 increases necrosis and apoptosis in xenograft tumours

DMS 79 tumours exposed to S4 alone and in combination with cisplatin displayed a marked increase in necrotic area (P=0.017 and P=0.0018, respectively), whereby almost 50% of tumour area was comprised of necrosis in the combination therapy group (figure 5).

The proportion of apoptotic cells increased in tumours treated with S4 (P=0.0038) (figure 5) as seen in the *in vitro* observations. Tumours that received S4 combined with cisplatin did not show any increase in apoptosis. It is likely that this reflects the increased time before tumours in the combination group were harvested (as compared to S4 alone). The increased levels of necrosis in the S4 combined with cisplatin group point towards extensive cell death at an earlier time point. Proliferation (Ki67) was unaltered in tumours at the time of excision,

suggesting that an increase in apoptosis, rather than a decrease in proliferation, is the mechanism through which S4 exerts its anti-tumour effects.

Quantification of hypoxic regions from sections stained for pimonidazole revealed a decrease in the hypoxic tumour fraction in tumours receiving S4 combined with cisplatin. This suggests that the combination therapy has reduced the hypoxic regions in the tumour, resulting in a concomitant reduction in CA IX expression and increased necrosis.

Combining S4 with repeated cisplatin doses gives additional benefit in growth reduction

Repeatedly dosing mice every 10 days with cisplatin mirrors clinical administration and this maintained tumour stasis in DMS 79 xenograft tumours that were also receiving S4 (figure 6A). These tumours took 5.7 fold longer than control tumours to reach RTV3 (figure 6B, 6C) compared with 3.6 fold longer when only single doses of cisplatin were utilized (figure 4C). Dosing with cisplatin after completion of the 3 week schedule of S4 resulted in a subsequent response, which mirrored that observed when treating chemo-naive tumours with cisplatin alone (figure 6A). This indicates that no resistance to therapy was acquired during this treatment. For the whole extended duration, mice did not show any signs of ill health or weight loss. Weights remained extremely stable, showing slight increases of an average of 3% by the end of the study, suggesting that this combination of therapies is extremely well tolerated *in vivo*. COR-L24 xenograft tumours showed an exquisite sensitivity to S4, keeping tumour sizes at ~250 mm³ for the 4 week schedule of S4 (figure 6D). COR-L24 xenograft tumours were also more cisplatin responsive than DMS 79. However, treatment was discontinued after 4 dosing cycles, due to the renal toxicity associated with repeated dosing of cisplatin chemotherapy. Discontinuation of the dosing of S4 or cisplatin resulted in a rapid gain in tumour volume. The combination of therapies revealed a slightly better treatment response than single agents with COR-L24 tumour regression in 3 out of 4 mice showing a strong response. Combined therapies were well tolerated with weights on average 3% increased by the end of the study.

To validate how S4 contributed to tumour stasis, a cohort of COR-L24 tumour bearing mice at 250 mm³ was treated for 4 days with S4 and harvested on day 5. Size-matched COR-L24 tumours were used as controls. COR-L24 tumours did show central cores of necrosis indicative of a poor blood perfusion and the development of a hypoxic environment (figure 6E). Quantification revealed a significant reduction in CA IX expression in COR-L24 tumour xenografts in response to S4 treatment (figure 6F). Tumour-associated hypoxia levels were also reduced in response to S4 treatment but accompanied by high levels of variation between hypoxia levels in the control group. COR-L24 xenograft tumour necrosis, apoptosis, proliferation, hypoxia and CA IX expression levels returned to control levels by the end of the study (tumour volumes 1000mm³), which was on average 40 days after discontinuation of the S4, cisplatin or combination treatment (data not shown).

Discussion

These studies clearly highlight the benefit of addition of CA IX inhibitors to standard chemotherapeutic regimens for the treatment of SCLC, a disease that currently suffers such a dismal prognosis. The marked effect on SCLC cell growth of inhibiting CA IX *in vitro* is further substantiated by the striking reduction in tumour growth observed in SCLC xenograft tumours. This effect is particularly evident when CA IX inhibition is combined with cisplatin chemotherapy. The mechanism of action of CA IX inhibition in increasing apoptosis and reducing viable hypoxic tumour regions is particularly relevant to SCLC where hypoxia is extremely prevalent¹⁴ and associated with a poor prognosis⁴. This is further supported by high CA IX expression in SCLC tumours in hypoxic regions^{4, 14}. Additional data have highlighted that CA9 knockdown regulates signalling pathways associated specifically with SCLC¹⁵. Taken together with the data presented in this manuscript, strong evidence exists to support the addition of CA IX inhibition to current chemotherapeutic approaches for treating SCLC.

In order to inhibit CA IX in SCLC cells, a member of the sulfamate class of CA IX inhibitors, S4, was utilised²². The specificity of S4 has been demonstrated in HT29 colorectal cancer

cells, whose response to S4 is attenuated following siRNA knockdown of CA IX (data not shown). Adding S4 to cells *in vitro* led to reduced viability, particularly when combined with cisplatin. The combination treatment was more effective under hypoxic than normoxic conditions, although cell death was still evident under normoxic conditions.

Doses of cisplatin utilised to reduce viability by 50% were between 3.0 and 6.9 μ M for DMS 79 and COR-L24 cells. In the literature, IC₅₀ values for cisplatin in a panel SCLC cell lines, including COR-L24, ranged from 0.11 to 3 μ M²³. However, in the previous study, cells were exposed to cisplatin for 8 days in comparison with the 2 day exposure presented in this manuscript, which could explain the lower IC₅₀ values. Another study that treated NSCLC cell lines with cisplatin for 2 days found IC₅₀ values to be 23.4 μ M for A549 cells and 18 μ M for Calu-1 cells²⁴. These values demonstrate the relative sensitivity of DMS 79 and COR-L24 cells, which is expected of cells that have not had prior exposure to platinum therapy. The concentrations of S4 required to induce a response are lower than alternative CA IX inhibitors, such as DH348 (1mM *in vitro*)²⁵.

The anti-tumour effects of combining S4 with cisplatin *in vitro* were verified by results *in vivo*. In DMS 79, S4 significantly reduced tumour growth when given alone, having comparable efficacy to cisplatin. Tumour growth delay was markedly enhanced when S4 was given along with cisplatin and was remarkably well tolerated. Multiple treatment cycles of the combination significantly enhanced the tumour growth delay in DMS 79. In COR-L24, both S4 and cisplatin alone were as effective as the combination therapy. Cisplatin was given as a one off dose of 3mg/kg based upon previous studies whereby 2-6mg/kg have been well tolerated whether alone or in combination studies²⁶. S4 doses were comparable with previous experiments utilising the alternative CA IX inhibitor, SLC-0111 at 50 mg/kg²⁷.

In the current study, both DMS 79 and COR-L24 SCLC cells showed a marked increase in apoptosis following treatment with S4 *in vitro*, particularly under hypoxic conditions. This suggests that the induction of apoptosis contributes to the cell sensitivity observed; a theory

that is further substantiated by the fact that DMS 79 xenograft tumours displayed elevated levels of apoptosis following treatment with S4. The finding that S4 induces apoptosis is consistent with previous studies showing that inhibition of CA IX induces apoptosis in renal and cervical cancer cell lines^{28, 29} and increases the response to pro-apoptotic drugs in hepatocellular carcinoma³⁰. The use of S4 within a laryngeal tumour model, however, showed no induction of apoptosis or necrosis³¹.

S4 alone enhances levels of apoptosis and although this was not observed with combination therapy, this is probably because of the timescale for collecting the tumours. Cisplatin treatment has been shown to induce apoptosis in a murine model of NSCLC³²; however, this response was only detected up to five days after therapy, at which point apoptosis reduced to baseline. Treating SCLC tumours with the combination of S4 and cisplatin likely resulted in enhanced apoptosis in the days immediately following cisplatin exposure. At the time of tumour excision, an average of 18 days after cisplatin administration, early apoptosis was probably observed as enhanced necrosis. Although in cervical and breast cancer cells, CA IX inhibition reduces proliferation^{22, 28, 33}, this was not found in SCLC xenograft tumours. There was no difference in proliferation between any treatment groups in DMS 79 tumours, which further supports the evidence that the effects of S4 and cisplatin are orchestrated through enhanced apoptosis as opposed to reduced proliferation.

DMS 79 tumours exposed to S4 in combination with a single dose of cisplatin showed enhanced necrosis, a reduction in pimonidazole staining and reduced CA IX expression. Reduced levels of pimonidazole are a marker of hypoxic cell death, as demonstrated previously using hypoxia-targeted agents against mouse-derived fibrosarcoma tumours³⁴. Therefore, the reduction in pimonidazole staining in the SCLC tumours suggests that the hypoxic fraction of tumours has been targeted by combination therapy. Given the recognized therapeutic resistance of hypoxic cells, this would bode well for subsequent treatment such as other chemotherapies or radiation, which is generally more effective under normoxic conditions. Cisplatin resistance within hypoxic environments has been previously

demonstrated in hepatocellular carcinoma cells *in vitro*³⁵. Subsequent addition of hypoxia-targeted agents to cisplatin to target hepatic tumours *in vivo* reduced tumour growth significantly³⁵. This response was comparable to that in the current study where inhibition of SCLC xenograft tumour growth following cisplatin and S4 treatment was indicative of combination therapy targeting hypoxic regions.

The reduction in CA IX protein levels following S4 administration was also apparent *in vitro* and is anticipated to be a result of those cells that express the greatest quantities of CA IX being targeted by the therapy, leading to a reduced overall expression. This is in line with the reduced CA IX expression found in tumours that had been exposed to S4. Furthermore, a recent study has described increased CA IX ectodomain shedding from colorectal carcinoma cells following treatment with S4, which correlated with reduced cell surface expression³⁶. SCLC cells could therefore be shedding CA IX following S4 treatment, decreasing CA IX detection in cells lysates.

In the past, single agent cisplatin treatment has shown anti-tumour properties in SCLC xenografts when given as multiple doses of 1-4mg/kg³⁷. Given the tolerability of S4/cisplatin combinations in the current study, mice bearing DMS 79 xenografts were subsequently exposed to repeated doses of cisplatin combined with S4. This more clinically relevant dosing strategy reduced tumour growth even more substantially, with tumour stasis observed throughout the period of combined treatment with S4. The period of time taken for tumours to reach maximum volume was 5.7 times that observed in control tumours. Once again, treatment did not cause any obvious adverse side-effects or weight loss. Importantly, when mice were dosed again with cisplatin after the end of S4 treatment, the tumour response mirrored that of mice treated with single doses of cisplatin. This is an important observation as in previous studies, repeated cisplatin doses in a murine model of NSCLC resulted in a cisplatin-resistant cell population³². Tumours stopped responding after a maximum of 4 doses of cisplatin due to enhanced DNA repair resulting in reduced apoptosis. The data presented in this manuscript suggest that cisplatin response was maintained throughout the multiple dose

cisplatin schedule with no evidence of acquired resistance, a significant issue that is well recognised in SCLC.

The ability of S4 to induce such a profound impairment in the growth of SCLC tumours without causing weight loss or obvious toxicity in mice is extremely encouraging as many molecular-targeted therapies that have entered clinical trials for SCLC, including Src kinase inhibition³⁸ and thalidomide³⁹, have all failed, largely due to off-target toxicity. This is a particular problem in SCLC patients who already rely heavily on cisplatin, which is itself highly toxic and causes nephrotoxicity in around one third of patients⁴⁰.

In conclusion, combining CA IX inhibition with cisplatin chemotherapy results in dramatically reduced tumour growth and enhanced apoptosis, without notable toxicity. Targeting CA IX therefore presents a novel, effective method of improving response to cisplatin chemotherapy that should be considered for the treatment of SCLC.

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Figure 1: Up-regulation of CA IX protein expression in hypoxic conditions and subsequent reduction with S4 treatment. (A) Growth characteristics of suspended DMS 79 and COR-L24 cell cultures. (B) CA IX expression in DMS 79 cells during 24 hours exposure to hypoxic (0.1% O₂) conditions. (C) CA IX expression in DMS 79 and COR-L24 cells incubated under hypoxic or normoxic conditions for 24 hours, with and without the addition of 30µM S4. Western blot results represent three independent experiments carried out in triplicate and graphs show mean ± SEM. As the running time of the COR-L24 samples on the polyacrylamide gel was somewhat longer than those of the DMS 79, this allowed for the discrimination of two isoforms of CA IX. Stars highlight differences from 0 hour expression or normoxic, control treatment unless otherwise indicated. * P < 0.05; ** P < 0.01.

Figure 2: Reduction in cell viability induced by treatment with S4 and cisplatin. DMS 79 (A) and COR-L24 (B) cells were treated with 100µM S4 and 7µM cisplatin alone and in combination under normoxic (20% O₂) and hypoxic (0.1% O₂) conditions. Viability was assessed after 48 hours using the Cell Titer-Glo viability assay. Viability of control-treated cells was set as 100%. Graphs show mean ± SEM and represent 3 separate experiments carried out in triplicate. Stars highlight differences from control unless annotations indicate otherwise * P < 0.05; ** P < 0.01; *** P < 0.001.

Figure 3: Increased apoptosis following treatment with S4 and cisplatin *in vitro*. DMS 79 cells were exposed to therapy for 36 hours and levels of apoptosis visualised 24 hours later using TUNEL analysis. Because of the hypoxia-sensitivity of COR-L24 cells, they were exposed to therapy for 24 hours before using TUNEL analysis. (A) Representative pictures of

apoptotic cells (green) and dapi (blue) in each treatment group. Photographs were taken at 40x magnification. (B) The proportion of apoptotic cells after therapy was quantified in Image J by measuring the area covered by FITC relative the DAPI labelling. Mean \pm SEM are shown and represent 3 experiments carried out in triplicate. Stars highlight significance compared with control cells under the same condition unless otherwise indicated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4: SCLC xenograft tumour growth delay as a result of S4 and cisplatin treatment. (A) Tumour growth in mm^3 following IP injection of S4, cisplatin, or both agents combined in DMS 79 xenograft tumours from the start of treatment. Horizontal arrows show the 5 day on, 2 day off dosing regimen of 50mg/kg S4. C and upwards facing arrows highlight 3mg/kg cisplatin doses. (B) Days taken for DMS 79 tumours to reach a relative tumour volume (RTV) three times that at the beginning of treatment. (C) Kaplan-Meier plot shows the percentage of tumours under RTV3 throughout the study. Graphs display mean \pm SEM, with each group containing 4-6 mice. Stars highlight differences from control unless otherwise annotated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 5: Changes in necrosis, apoptosis, proliferation, hypoxia and CA IX expression in DMS 79 tumours following treatment. (A) Central sections of tumours were stained with H&E, for apoptosis (TUNEL[green]), proliferation (Ki67[brown]), hypoxia (pimonidazole [pimo][brown]) and CA IX (brown) following treatment of mice bearing DMS 79 xenograft tumours with vehicle, S4 and S4 with cisplatin. Nuclei are counterstained using haematoxylin. Arrows highlight necrosis in H&E pictures. (B) Necrosis, apoptosis and proliferation were quantified using HistoQuant Analysis within Panoramic Viewer. Hypoxia and CA IX were quantified in Image J using the Color Deconvolution plugin. Graphs display mean \pm SEM and represent 5-7 tumours in each treatment group. Stars signify differences from control-treated tumours unless otherwise indicated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Original magnifications are 100x for TUNEL and Ki67 and 40x for pimo and CA IX.

Figure 6: Tumour growth reduction when using multiple cisplatin doses combined with S4. (A) DMS 79 xenograft tumour growth following the start of IP injections. Horizontal arrows show the ‘5 day on, 2 day off’ dosing regimen on 50mg/kg S4. Upwards facing arrows with “C” highlight 3mg/kg cisplatin doses administered every 10 days. (B) Days taken for tumours to reach a relative tumour volume (RTV) three times the volume at the beginning of treatment. (C) Percentage of mice bearing tumours below RTV3 following treatment. (D) COR-L24 xenograft tumour growth following the start of IP injections. Depicted are the individual COR-L24 tumour growth curves of the individual mice in the control, S4 & cisplatin alone and combination groups. COR-L24 xenograft tumours therapy is indicated in the graphs with 50mg/kg S4 on ‘5 day on, 2 day off’ dosing regimen for 4 cycles and cisplatin every 10 days (4 times). (E) Central COR-L24 tumour sections stained with H&E, for apoptosis (TUNEL[green]), proliferation (Ki67[brown]), hypoxia (pimonidazole [pimo][brown]) and CA IX (brown) after 4 days of therapy with vehicle or S4 of mice bearing COR-L24 xenograft tumours (N=3 each). Images are serial sections of one control and S4 treated tumour which were chosen as they were most representative for the treatment group (F) Necrosis, apoptosis, proliferation and hypoxia were quantified using HistoQuant Analysis within Panoramic Viewer. Semiquantitative analysis of CA IX immunostaining performed according to Allred et al. (1993)⁴¹. Graphs display mean \pm SEM and represent 4-6 mice in each group. Stars highlight differences from control unless otherwise indicated. * P < 0.05.

Supplemental figure 1: CA IX expression in DMS 79 and COR-L24 cells incubated under hypoxic or normoxic conditions for 24 hours, with and without the addition of 100 μ M S4. Stars highlight significance compared with control cells under the same condition unless otherwise indicated. * P < 0.05; ** P < 0.01; *** P < 0.001.

Supplemental figure 2: Cell viability of COR-L24 cells assessed in vitro after 24 hours with the live/dead viability assay. The sum of live (Calcein, green) and dead (Ethidium homodimer-1, red) was set to 1. The viability was reduced by treatment with S4 under

normoxic (20% O₂) conditions. Graphs show mean \pm SEM and represent 2 separate experiments carried out in triplicate.

Figure 1

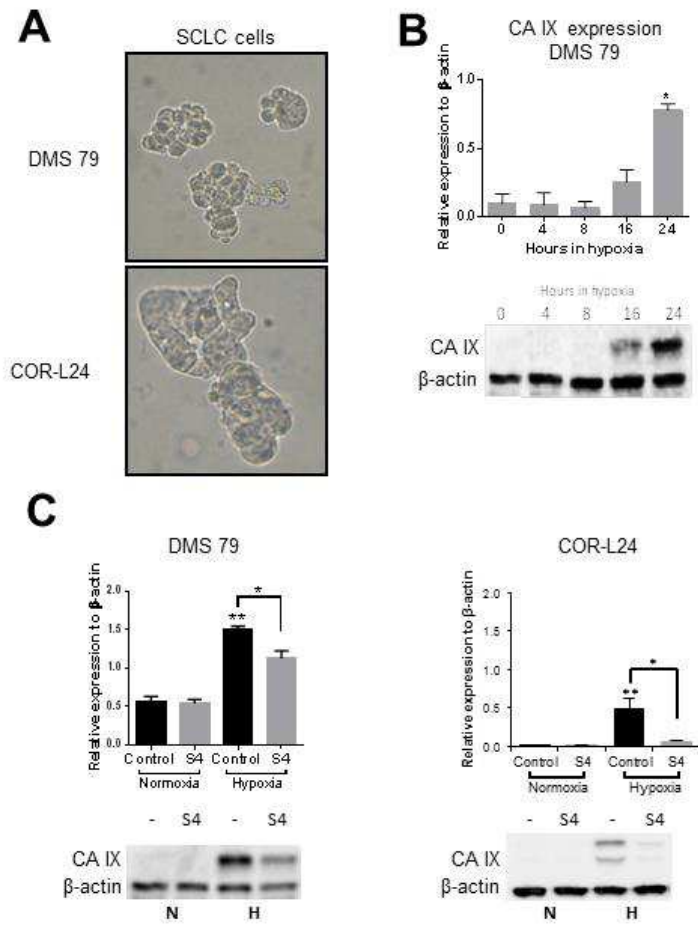


Figure 2

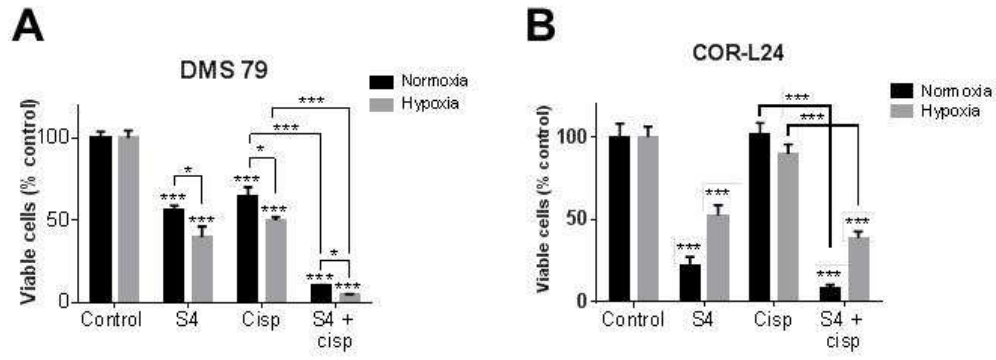


Figure 3

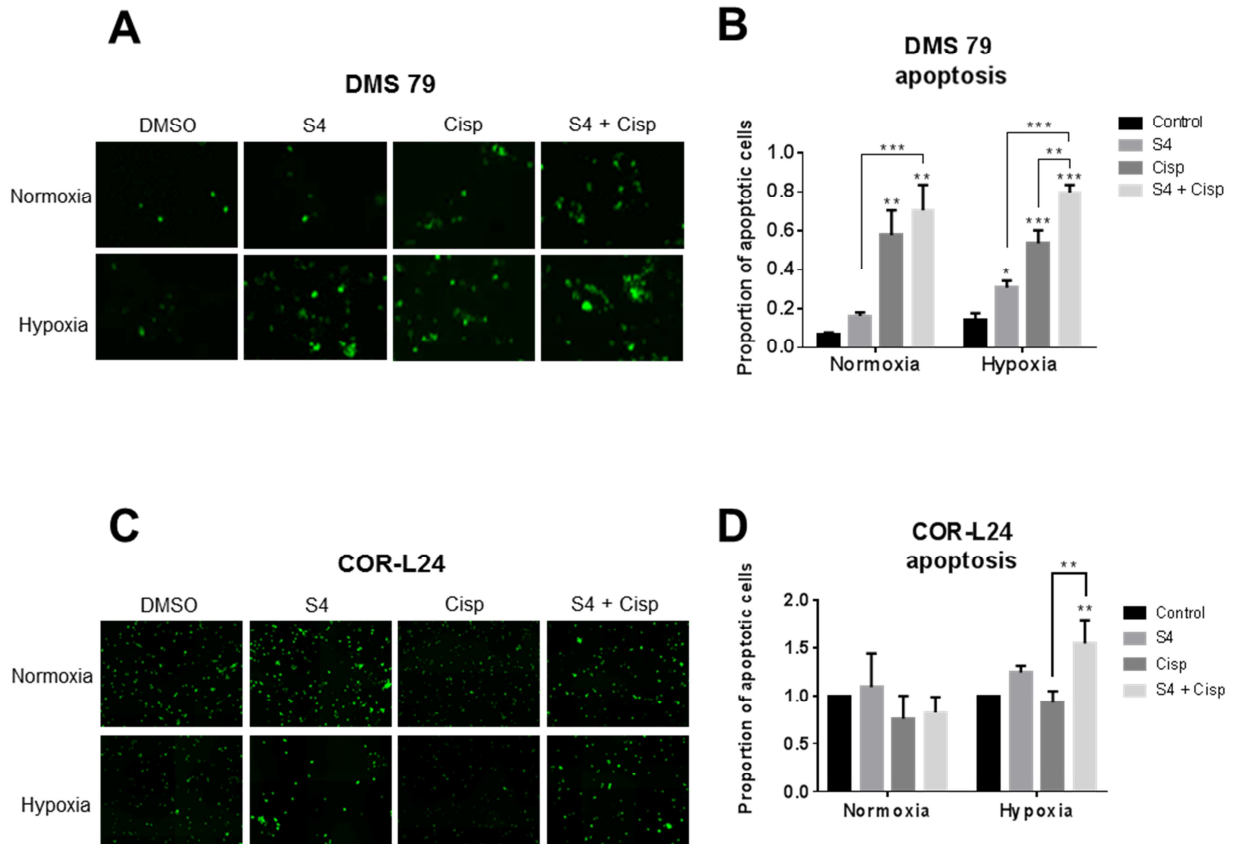


Figure 4

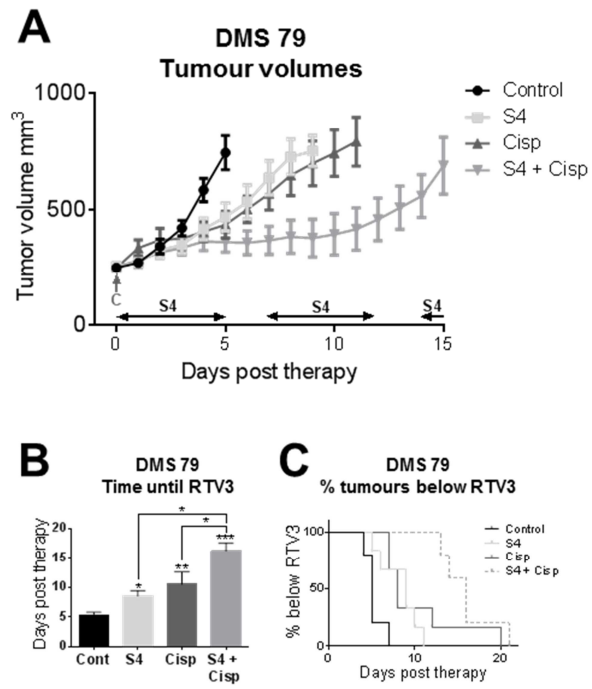


Figure 5

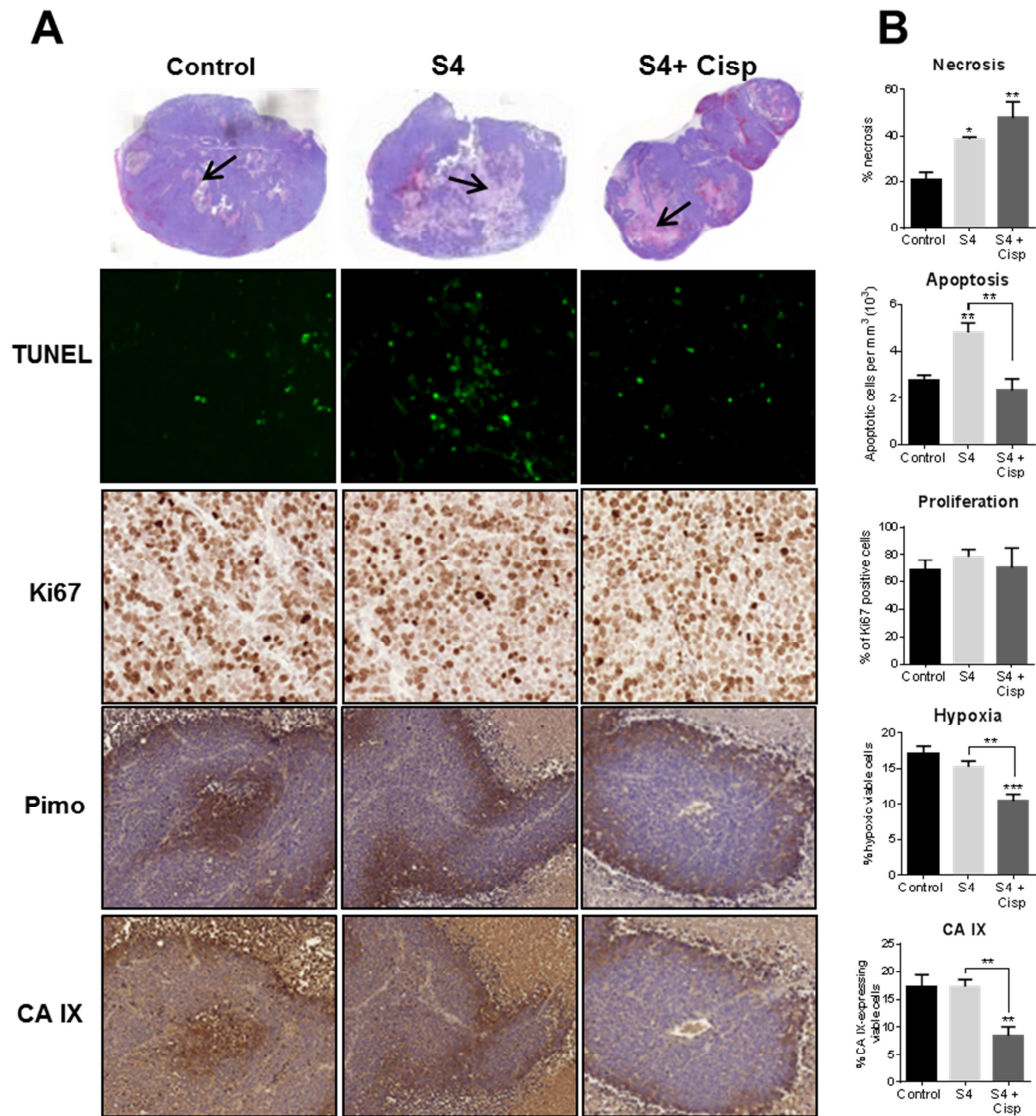


Figure 6

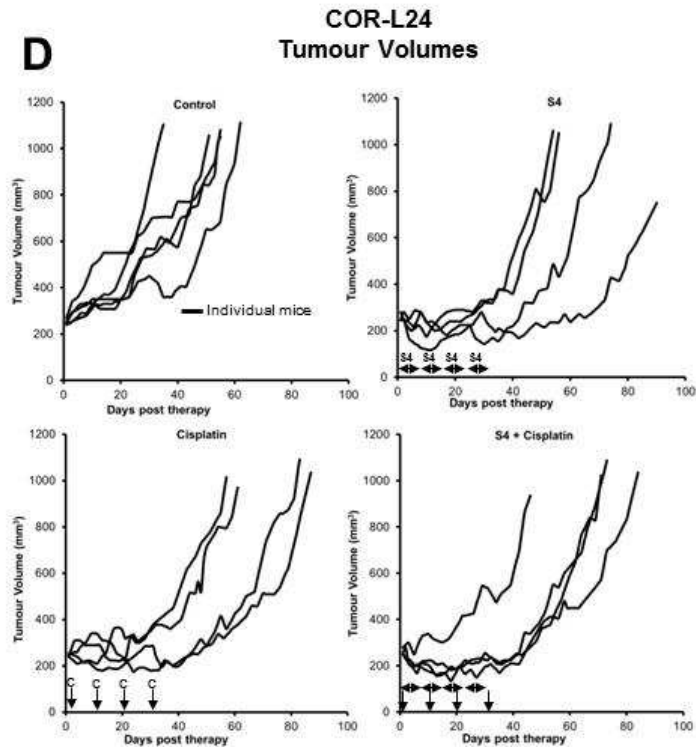
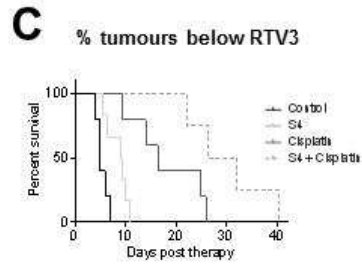
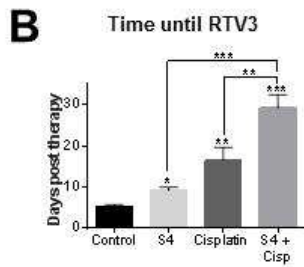
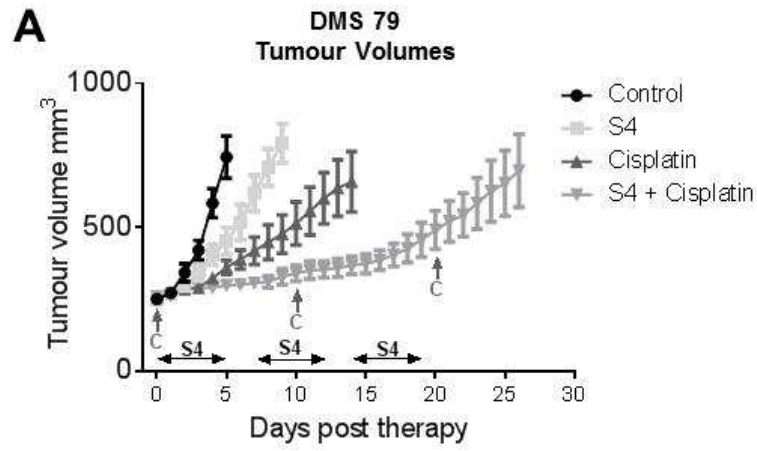
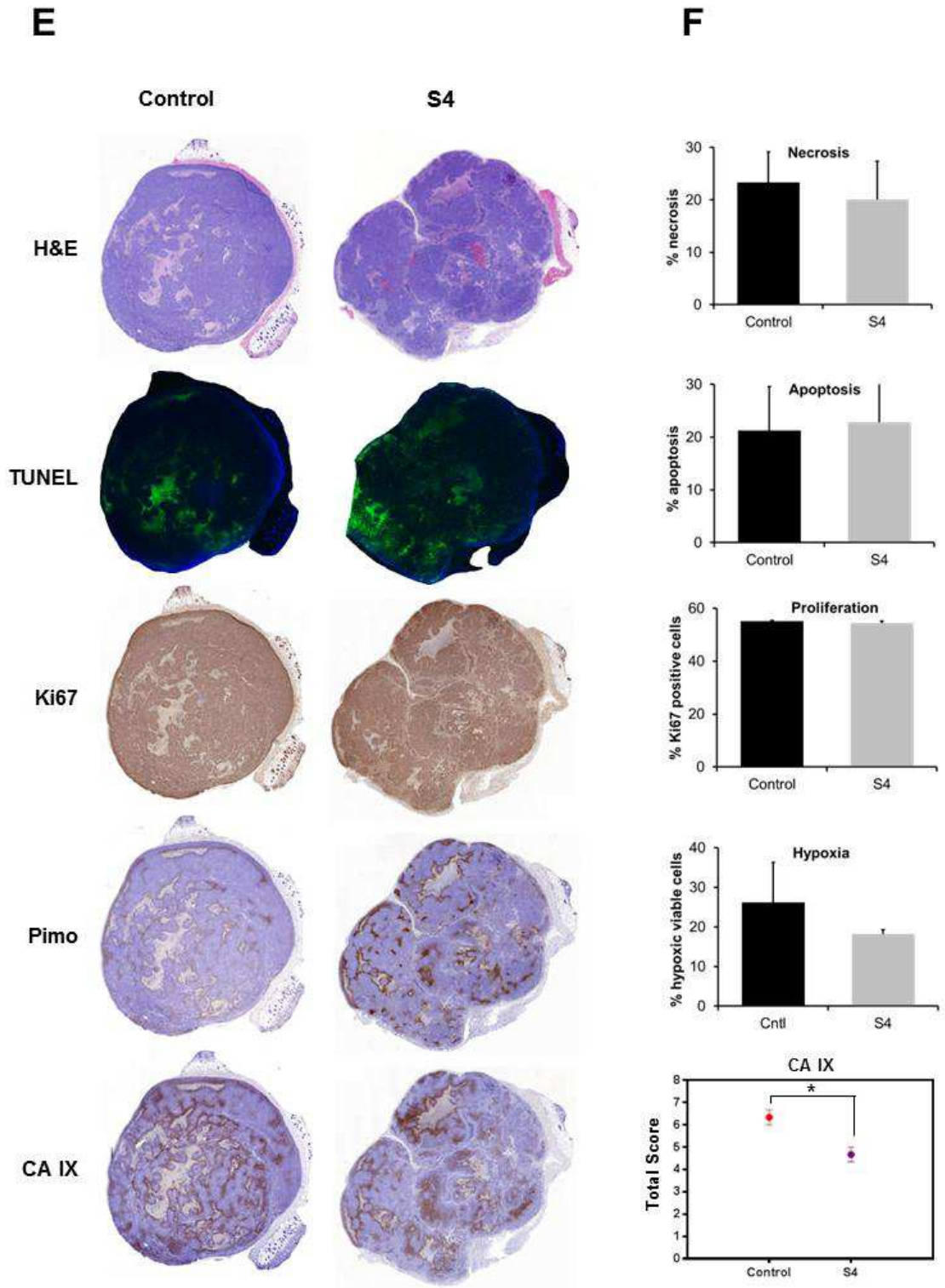
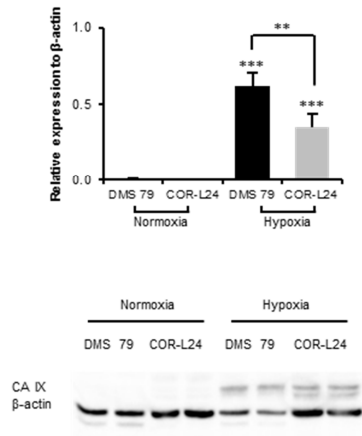


Figure 6, Cont'd



Supplemental figure 1



Supplemental figure 2

Live (green) / Dead (red) staining – COR-L24

