

## The $x_c^-$ cystine/glutamate antiporter as a potential therapeutic target for small-cell lung cancer: use of sulfasalazine

Jun Guan · Maisie Lo · Peter Dockery · Sarah Mahon · Cristina M. Karp ·  
Arthur R. Buckley · Stephen Lam · Peter W. Gout · Yu-Zhuo Wang

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### Abstract

**Purpose** To determine whether the  $x_c^-$  cystine transporter could be a useful therapeutic target for small-cell lung cancer (SCLC).

**Methods** Human SCLC cell cultures were examined for growth dependence on extracellular cystine,  $x_c^-$  expression, glutathione levels and response to highly specific  $x_c^-$  inhibitors, i.e., monosodium glutamate (MSG) and the anti-inflammatory drug, sulfasalazine (SASP). In studying tumor growth inhibition by SASP, use was also made of a novel SCLC tissue xenograft model, LU6-SCLC, derived from a chemoresistant patient's SCLC specimen.

**Results** Growth of NCI-H69 and NCI-H82 SCLC cells greatly depended on  $x_c^-$ -mediated uptake of cystine. SASP substantially reduced their glutathione levels (>70%; 0.3 mM SASP; 24 h) and growth (72 h) with  $IC_{50}$ s of 0.21 and 0.13 mM, respectively; MSG also inhibited growth

markedly. Both SASP- and MSG-induced growth arrests were largely prevented by cystine uptake-enhancing 2-mercaptoethanol (66  $\mu$ M) indicating they were primarily due to cystine starvation. Without major side-effects, SASP (i.p.) restrained growth of NCI-H69 cell xenografts (~50%) and, importantly, substantially inhibited growth of the clinically more relevant LU6-SCLC tissue xenografts (~70% by stereological analysis), reducing tumor glutathione contents.

**Conclusions** The  $x_c^-$  cystine/glutamate antiporter is potentially useful as a target for therapy of SCLC based on glutathione depletion. Sulfasalazine may be readily used for this approach, especially in combination chemotherapy.

**Keywords**  $x_c^-$  transporter · Cystine · Glutathione · Small-cell lung cancer · SCLC xenograft model · Sulfasalazine

J. Guan · P. W. Gout (✉) · Y.-Z. Wang (✉)  
Department of Cancer Endocrinology,  
BC Cancer Agency, Research Centre,  
675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada  
e-mail: pgout@bccrc.ca

Y.-Z. Wang  
e-mail: ywang@bccrc.ca

M. Lo  
Department of Cancer Genetics, BC Cancer Agency,  
Research Centre, Vancouver, BC V5Z 1L3, Canada

P. Dockery  
Department of Anatomy,  
National University of Ireland, Galway, Ireland

S. Mahon  
Department of Surgery,  
National University of Ireland, Galway, Ireland

C. M. Karp  
Center for Advanced Biotechnology and Medicine,  
Rutgers University, Piscataway, NJ 08854, USA

A. R. Buckley  
James L. Winkle College of Pharmacy,  
University of Cincinnati Academic Health Center,  
Cincinnati, OH 45267-0004, USA

S. Lam  
Department of Cancer Imaging, BC Cancer Agency,  
Research Centre, Vancouver, BC V5Z 1L3, Canada

Y.-Z. Wang  
The Prostate Centre at Vancouver General Hospital  
and Department of Urologic Sciences, University of BC,  
Vancouver, BC V6T 2B5, Canada

## Introduction

Lung cancer is the leading cause of cancer deaths worldwide. It is difficult to detect and patients often present at an advanced stage [1]. Small-cell lung cancer (SCLC) is an aggressive form of the disease, characterized by rapid growth and early metastasis, representing 13–20% of all lung cancers. Although SCLC is initially responsive to chemotherapy and radiation, a large variety of multimodality treatments have failed to control or cure this disease in most patients, including combination chemotherapy based on cisplatin and etoposide, commonly used in North America for SCLC treatment. Thus, most SCLC patients become resistant to chemotherapy, relapse, and die from the disease, with over 95% of patients dying within 5 years of diagnosis [2, 3]. Current chemotherapies are hence not adequate and new therapeutic approaches are critically required.

Cystine/cysteine starvation leading to glutathione (GSH) depletion in target cells, based on inhibition of the  $x_c^-$  cystine/glutamate antiporter, has been suggested by us as a potential therapeutic approach for a variety of cancers [4–8]. The  $x_c^-$  plasma membrane transporter mediates exchange of extracellular cystine (the oxidized form of cysteine) for intracellular glutamate with a stoichiometry of 1:1 [9]. There is an increasing amount of evidence suggesting that it has an important role in uptake of cystine/cysteine by cancers that depend for growth and viability on supply of the amino acid from the microenvironment [4–8, 10, 11]. Thus the  $x_c^-$  transporter has a key role in the *in vivo* secretion by somatic cells (e.g., activated macrophages, dendritic cells) of cysteine, which can be readily taken up by neighboring cells via e.g., the universally expressed ASC transport system [11, 12]; in contrast, cystine is not readily taken up by cells and  $x_c^-$  can, if expressed by cancer cells, directly mediate their uptake of cystine [11]. Intracellularly, cystine is rapidly reduced to cysteine, essential for biosynthesis of protein and in particular as a rate-limiting substrate of GSH, a tripeptide thiol which plays an essential role in cellular defenses against oxidative stress [13, 14]. GSH has a short half-life and intracellular cysteine depletion can readily lead to GSH depletion with subsequent growth arrest and reduced defense against oxidative stress [13]. Inhibition of the  $x_c^-$  transporter leading to cystine/cysteine starvation and subsequent GSH depletion therefore represents a potential therapeutic approach for malignancies dependent on extracellular cystine/cysteine [11].

In a search for a relatively non-toxic inhibitor of the transporter, we have previously shown that sulfasalazine (SASP), an anti-inflammatory drug routinely used in clinical therapy of inflammatory bowel disease and

rheumatoid arthritis, is a potent  $x_c^-$  inhibitor [5]. As demonstrated, SASP-induced  $x_c^-$  inhibition can readily lead to cystine starvation of a variety of experimental cancers, including lymphoma, prostate, and breast cancer cell lines, with subsequent reduction of intracellular GSH levels and growth arrest *in vitro* and *in vivo* [5, 7, 15].

Preclinical cancer models, based on xenografts of cultured cancer cell lines, in general do not adequately represent the disease as it is presented in the clinic, since they lack the tumor heterogeneity typical for the malignancy and often do not properly predict efficacy of anticancer agents in clinical therapy [16, 17]. This instigated us to develop new cancer models with improved clinical relevance based on grafting of patients' cancer tissue into the subrenal capsule graft site of NOD-SCID mice. This graft site allows high tissue perfusion and potentially rapid development of graft microvasculature and its use has been shown to result in very high tumor take rates (>95%) coupled to major retention of phenotypic and genotypic properties in contrast to other graft sites such as the commonly used subcutaneous compartment [18, 19]. From a variety of primary human lung cancers we have recently developed more than 50 transplantable tumor tissue lines which resemble the original cancers in major histopathological and genotypic features. One of these tumor tissue lines, LU6-SCLC, was generated from biopsy tissue of a patient's SCLC which was resistant to multiple chemotherapy drugs. This SCLC tissue line is considered to have greater clinical relevance than conventional SCLC cell lines [19].

In the present study we have used the transplantable LU6-SCLC tumor tissue line to examine whether SASP has potential for application in lung cancer therapy. In addition we used well-established cultured human SCLC cell lines, NCI-H69 and NCI-H82, to study SCLC cell growth requirements for extracellular cystine, expression of the  $x_c^-$  cystine transporter and sensitivity to SASP. We could not use the LU6-SCLC tissue line to fully examine these properties, since tumor tissue lines, like tumors, are heterogeneous, consisting of a variety of cancer cell subpopulations and normal stroma cells with varying growth requirements, and LU6-SCLC tissue could hence not be expected to survive as an intact population under ordinary *in vitro* conditions [18]. It was found that both NCI-H69 and NCI-H82 cell lines were highly sensitive to SASP *in vitro*, with GSH depletion and subsequent growth arrest resulting from cystine starvation induced by  $x_c^-$  inhibition. *In vivo*, SASP substantially inhibited growth of NCI-H69 and LU6-SCLC xenografts without major toxicity to the mouse hosts. Overall, the data indicate that the  $x_c^-$  cystine/glutamate antiporter is potentially useful as a therapeutic target for SCLC.

## Materials and methods

### Materials and animals

Chemicals, stains, solvents, and solutions were obtained from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada, unless otherwise indicated. Eight- to ten-week old male NOD-SCID mice were bred by the BC Cancer Research Centre Animal Resource Centre, BC Cancer Agency, Vancouver, Canada. Animal care and experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

### Cell cultures

Human SCLC cell lines NCI-H69 and NCI-H82 were obtained from the American Type Culture Collection (Manassas, VA). Cell suspension cultures were maintained at 37°C in RPMI-1640 medium (StemCell Technologies Inc., Vancouver, BC, Canada), supplemented with 10% heat-inactivated fetal bovine serum (FBS; GibcoBRL Inc., Burlington, ON, Canada), 100 units/ml penicillin, and 100 µg/ml streptomycin (StemCell Technologies Inc.) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Cultures were monitored for growth characteristics and doubling times and maintained for not more than 20 passages. DU145, MDA231, and 184A1 monolayer cultures were maintained as previously described [6, 7].

### Patient-derived SCLC tissue line

Bronchial SCLC biopsy tissue, coded LU6-SCLC, was originally obtained at the British Columbia Cancer Agency (Vancouver, Canada) from a patient whose SCLC was resistant to multiple chemotherapeutic drugs. Informed consent was given following a protocol approved by the Clinical Research Ethics Board of the University of British Columbia. The LU6-SCLC tumor tissue line was developed by grafting the tissue into the subrenal capsule site of NOD-SCID mice and maintained by serial transplantation in such mice [19]. Xenografts of the 16th generation, used in this study, showed no histological differences compared to the second generation previously presented [19] (data not shown).

### Assessment of growth requirements for exogenous cystine

Single cell suspensions were obtained by aspiration (NCI-H69) or trypsinization (NCI-H82). Cells were then washed twice by centrifugation (4 min at ~100g) and resuspension into cystine/methionine-deficient RPMI-1640 medium (supplemented with 10% dialyzed FBS/antibiotics) and

500 µl aliquots were distributed into 12-well plates (Linbro, Flow Laboratories, Mississauga, ON, Canada). After 24 h of incubation, methionine (0.1 mM) was added and the cultures were further incubated with cystine (0.2 mM), cystathionine (0.15 mM) or cystine-deficient medium. Total volume 1.0 ml; initial cell concentration: NCI-H69, ~10<sup>5</sup> cells/ml; NCI-H82, ~12 × 10<sup>3</sup> cells/ml. On day 5, cells were harvested and their numbers were determined using an electronic cell counter (Beckman-Coulter, Hialeah, FL, USA). Results are presented as percentage growth (means ± SD) relative to controls (growth of the cultures in complete RPMI-1640 medium).

### Reverse transcription-polymerase chain reaction (RT-PCR)

Log phase cultures of NCI-H69 and NCI-H82 cells in maintenance medium were centrifuged and the cell pellets were snap frozen in liquid nitrogen for assay of *xCT*-mRNA expression by RT-PCR as previously described [7].

### Glutathione assay

Total GSH levels in cultured cells [(0.5–1) × 10<sup>6</sup> cells/sample] or xenograft tissue (50 mg; 3–7 samples) were determined (in triplicate) using an ApoGSH<sup>TM</sup> Glutathione Colorimetric Detection kit from BioVision Research Products (Mountain View, CA) and following the manufacturer's instructions [7].

### SASP solutions

Solubilization of SASP involved use of 0.1 N NaOH and adjustment of the pH to ~7.5 using 1 N HCl, as previously described [5]. Drug and vehicle solutions were prepared, filter sterilized, and assayed under subdued light conditions. For in vitro studies, fresh 10 mM SASP stock solutions were prepared and appropriately diluted with maintenance medium for immediate addition to cell cultures. For in vivo studies, 50 mM SASP solutions (pH about 8.0) were freshly prepared every day [5].

### Testing of SASP and monosodium glutamate (MSG) in vitro

Suspensions (600 µl aliquots) of NCI-H69 and NCI-H82 cells in minimum essential medium (MEM)/FBS(10%)/antibiotics were incubated in 24-well plates (Linbro) (MEM was used since it contains cystine at approximately physiological levels, as distinct from the much higher cystine levels in RPMI-1640 [5]). The next day, various concentrations of SASP, without or with 2-mercaptoethanol (2-ME; 66 µM), were added for a further 72 h incubation. Total volume, 700 µl; initial cell concentrations, 5 × 10<sup>3</sup> cells/well.

Cytotoxicity of SASP was determined via the hemacytometer-based Trypan blue exclusion assay. Percent viability was determined by counting the number of non-blue-stained cells relative to the total number of cells. The  $IC_{50}$  of SASP was determined via a 72 h incubation with a range of SASP concentrations. The effect of MSG on growth of the two cell lines was similarly tested, using initial cell concentrations of 10,000 cells/well.

#### Testing of SASP using NCI-H69 cell and LU6-SCLC tissue xenografts

To generate tumors, NCI-H69 cells were packed in polymerized collagen and grafted ( $2 \times 10^6$  cells/graft) under renal capsules (two grafts per kidney) of NOD-SCID mice, using routine procedures [19]. For SASP efficacy evaluation experiments, tumors (volume  $\sim 200 \text{ mm}^3$ ) were cut into  $3 \times 2 \times 1 \text{ mm}$  pieces for re-grafting under kidney capsules of male NOD-SCID mice (two grafts per kidney). When the tumors reached an average volume of about  $20\text{--}50 \text{ mm}^3$  (as determined in replicate mice), the animals were sorted into control and treatment groups (5 mice/group) and received, under subdued light conditions, intraperitoneal (i.p.) injections (every 12 h) of saline (controls) or SASP (250 mg/kg body weight) for periods up to 14 days. At the end of the treatment, animals were sacrificed and tumors were harvested, measured and sections were prepared for histological analysis, as previously described [19]. Tumor size was expressed in cubic millimeter, using the formula: volume ( $\text{mm}^3$ ) =  $0.52 \times \text{length} \times \text{width} \times \text{height}$  (in mm). Data are presented as means  $\pm$  SEM. Fresh SASP solutions were prepared every day. Food and water were provided to the mice ad libitum throughout the experiment. A similar protocol was used to investigate the effect of SASP on LU6-SCLC xenografts. In this case, tumors were also examined for GSH content and subjected to stereologic analysis.

#### Stereology

Following harvesting, LU6-SCLC xenografts of SASP-treated and control mice were fixed in 10% neutral buffered formalin, using routine procedures previously described [19] and then embedded in paraffin wax for light microscopy. To this end tissue was randomly orientated and allowed to settle haphazardly in wax. The subsequent sections were considered isotropic uniform and random in orientation (IUR). The tissue blocks were cut into  $5 \mu\text{m}$  sections using a rotary microtome. Sections were then stained with Haematoxylin and Eosin (H&E). A systematic random sampling strategy was employed throughout this study [20]. A series of micrographs was taken on a Leitz (DMRXE) microscope fitted with a DFC 300FX camera at an initial magnification of

$20\times$  and viewed using Adobe Photoshop©. Appropriate stereological grids [21] were applied to the images and the following features were recorded: number of blood vessels, points hitting blood vessels, and number of points in total hitting the reference section were recorded along with the final magnification. When all data had been collected the following parameters were calculated. *Total tumor volume*: total tumor volume was estimated via measurement of axial dimensions made on dissected tissue mass. *Volume fraction* ( $V_v$ ): the volume fraction of necrotic tissue in the tumor was estimated by simple point counting [20], absolute volumes were estimated by combining  $V_v$  with volumes. *Number per unit area* ( $N_a$ ): the numbers of blood vessel profiles were counted using an unbiased counting frame. This was divided by the area of the sampling frame to obtain the  $N_a$ . *Length density* ( $L_v$ ): length density, i.e., length of blood vessel per unit volume of tissue, was obtained by multiplying the  $N_a$  estimates by two. *Diffusion distance* ( $R_d$ ): the radial diffusion distance provides a potentially useful physiologically relevant parameter, i.e., a description of a radius of oxygen diffusion around a blood vessel. The value is estimated as follows:  $1/\sqrt{(L_v \times \pi)}$  [22]. *Total length*: as the total volume of the tissue was already known, the total length of blood vessels could be calculated using the equation:  $L_v \times \text{total volume}$ .

#### Statistical analysis

Values for each individual animal were used to derive group means and standard errors (SEM). Ratio estimates were logarithmically transformed prior to testing. The Student's  $t$  test was used to determine statistical significance; results with a  $P \leq 0.05$  were considered significant. In addition, ANOVA was used to compare tumor volumes in SASP-treated and control mice.

## Results

#### Growth requirements for extracellular cystine in vitro

Cysteine can be generated in tissues (e.g., liver) by methionine metabolism through the trans-sulfuration pathway involving cleavage of cystathionine by  $\gamma$ -cystathionase to  $\alpha$ -ketobutyrate and cysteine [23]. In contrast, certain types of cancer cells are not able to adequately generate cysteine and, to sustain growth and viability, require uptake of the amino acid from their micro-environment. To determine if NCI-H69 and NCI-H82 lung cancer cells could proliferate in the absence of extracellular cystine, the two cell lines were cultured in medium from which cystine had been specifically omitted. In addition it was determined whether cystathionine could act as a substitute for cystine. Whereas the H69 and

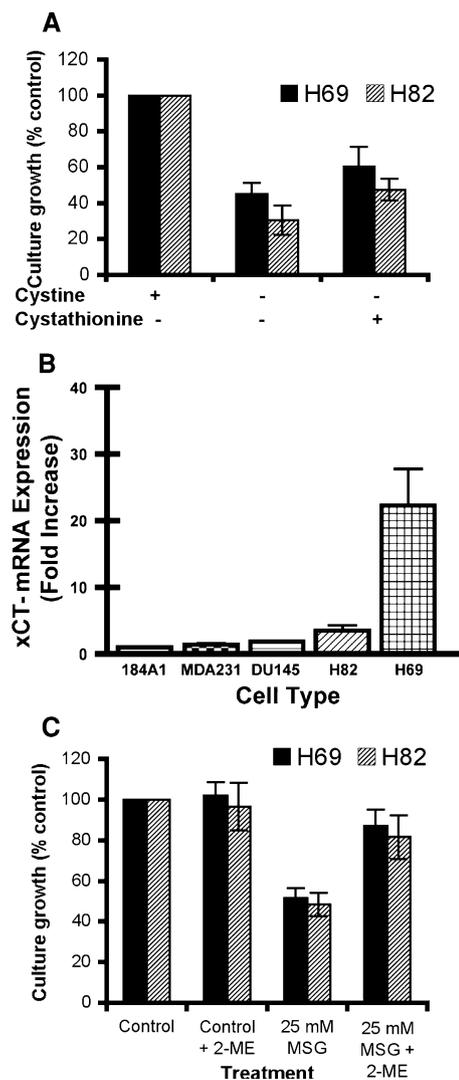
H82 cell lines grew actively in complete culture medium (containing 10% dialyzed FBS), specific omission of cystine from this medium led in both cases to a substantial reduction of culture growth, i.e., 55 and 70% for H69 and H82 cells, respectively (Fig. 1a). Furthermore, cystathionine, which can fully replace cystine in certain cell cultures [24], could not completely act as a cystine substitute. However, it stimulated growth of the two cell lines somewhat in the absence of exogenous cystine, indicating presence in the cells of some  $\gamma$ -cystathionase activity. Taken together, the results indicate that NCI-H69 and NCI-H82 cells have some cysteine-producing ability, but cannot synthesize adequate amounts of the amino acid and hence are dependent on uptake of cystine/cysteine from their micro-environment.

#### Expression of the $x_c^-$ cystine transporter

The  $x_c^-$  cystine/glutamate antiporter is a heterodimeric plasma membrane protein consisting of a heavy subunit, 4F2hc, and a light subunit,  $xCT$ , linked by a disulfide bridge. The 4F2hc subunit is a common component of heterodimeric amino acid transporters with a role in anchoring the heterodimer to the plasma membrane; the  $xCT$  subunit controls cystine transport function and specificity [25]. Since the 4F2hc subunit is generally present in cells, only expression of the cystine-specific  $xCT$  subunit was examined. As shown in Fig. 1b, NCI-H82 and in particular NCI-H69 cells expressed the  $xCT$  gene, as measured via RT-PCR, to a significantly greater extent than other established cell lines such as the DU145 prostate cancer [7], MDA231 breast cancer and 184A1 immortalized, normal breast [6] cell lines. The higher  $xCT$  expression by the H69 cells, relative to the H82 cells ( $P < 0.01$ ), suggests that they have more  $x_c^-$  cystine transporters per cell than the H82 cells and hence may have a greater cystine uptake capability.

#### Effect of MSG on culture growth

MSG is the main exchange substrate in the  $x_c^-$ -mediated uptake of cystine, with an affinity for  $x_c^-$  similar to that of cystine; as such MSG is a potent, highly specific inhibitor of cystine uptake via the  $x_c^-$  transporter [11]. MSG inhibited growth of NCI-H69 and NCI-H82 cultures in MEM supplemented with FBS (10%) with a maximal growth reduction of about 53% (data not shown). As shown in Fig. 1c, the growth inhibition by MSG (25 mM) was largely prevented by inclusion in the medium of 66  $\mu$ M 2-ME, a compound commonly used to shuttle cystine into cells via the L-transport system thus circumventing inhibition of  $x_c^-$  transporter function [4–8, 11, 26]. The MSG-induced growth inhibition was of the same order as the reduction in growth observed when cystine was specifically



**Fig. 1** Growth requirement of NCI-H69 and NCI-H82 SCLC cell cultures for extracellular cystine;  $x_c^-$  expression. **a** Effect on culture growth of omission of cystine from the culture medium and substitution of cystine by cystathionine. Cells were incubated for 72 h in RPMI-1640 medium supplemented with dialyzed FBS (10%) and antibiotics in (1) the presence of cystine (0.2 mM); control, (2) absence of cystine and (3) absence of cystine and presence of cystathionine (0.15 mM), as described in “Materials and methods”. Cell numbers were determined using an electronic cell counter. Data are representative of results from three experiments and are expressed as percentage of control growth (mean  $\pm$  SD). **b** Expression of  $xCT$ -mRNA in NCI-H69 and NCI-H82 cells, as shown by RT-PCR, in comparison with 184A1 immortalized normal breast, MDA231 breast cancer and DU145 prostate cancer cells. The  $xCT$  subunits were normalized to levels of  $\beta$ -actin-mRNA within a sample (triplicates) and the mean levels of expression normalized to that of 184A1. Data are presented as means  $\pm$  SEM and are representative of results from two experiments. **c** Growth inhibition by MSG (25 mM) of NCI-H69 and NCI-H82 SCLC cell cultures in MEM supplemented with FBS (10%) and antibiotics (72 h): prevention by 2-mercaptoethanol (2-ME; 66  $\mu$ M). Cell viability and growth were assayed using a trypan blue exclusion assay (triplicate). Data are expressed as number of viable cells relative to control, means  $\pm$  SD, and are representative of results from three experiments

omitted from the medium (see Fig. 1a). Taken together, the data indicate (1) that the growth reduction of the cell lines by MSG was due to inhibition of the  $x_c^-$  transporter leading to exclusion of extracellular cystine and (2) that the  $x_c^-$  transporter for both cell lines is the main route for uptake of extracellular cystine for regular growth.

#### Effect of SASP on culture growth

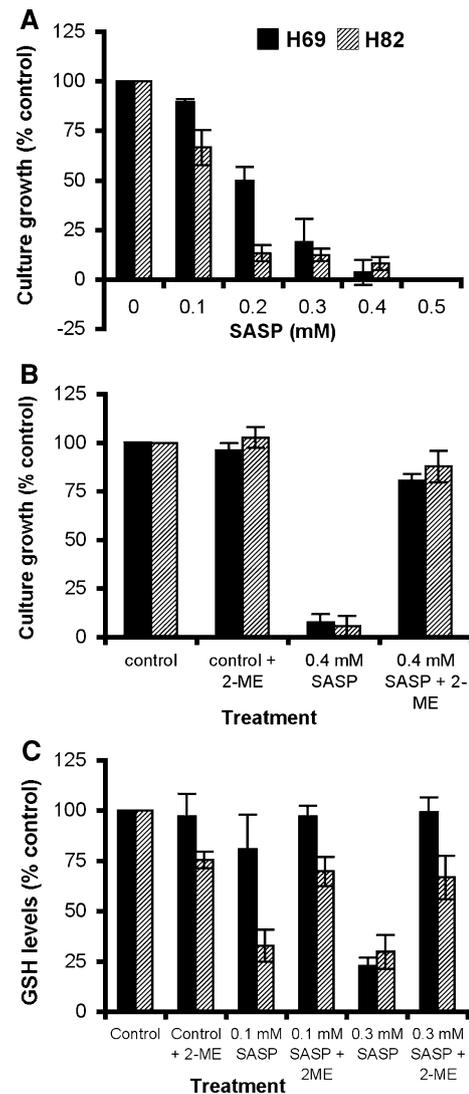
SASP has shown marked growth-inhibitory activity, based on  $x_c^-$  inhibition, in a variety of systems [5–7, 11]. Here it inhibited growth of NCI-H69 and NCI-H82 cultures in MEM supplemented with FBS (10%) with  $IC_{50}$ s of 0.21 and 0.13 mM, respectively (Fig. 2a). At 0.3 mM SASP, growth of both H69 and H82 cell lines was inhibited by ~80%; essentially complete growth inhibition of the cultures was obtained at 0.4 mM SASP. In the SASP concentration range 0.1–0.2 mM, the H82 cells were more sensitive to SASP than the H69 cells. As shown in Fig. 2b, growth inhibition by 0.4 mM SASP could be largely prevented by inclusion in the medium of 66  $\mu$ M 2-ME. This indicates that the SASP-induced growth arrest of both cell lines was primarily due to cystine starvation.

#### Effect of SASP on intracellular GSH levels in vitro

As shown in Fig. 2c, a 24 h incubation of NCI-H69 and NCI-H82 cells with SASP (0.1 and 0.3 mM) led to marked reductions in their intracellular total GSH levels. At 0.3 mM SASP the GSH levels in H69 and H82 cells decreased to 22.7 (SD = 0.04) and 29.8% (SD = 0.08) of normal values, respectively, i.e., a reduction >70%. Furthermore, the SASP-induced reduction in GSH levels was completely (H69) or substantially (H82) prevented by 66  $\mu$ M 2-ME, indicating that it was mainly due to cystine starvation. As indicated by the Neutral Red cytotoxicity assay, the cells treated with 0.3 mM SASP for 24 h in the absence of 2-ME were mostly viable showing 99 and 67% viability for H69 and H82 cells, respectively (data not shown). The extensive reduction in GSH levels induced by SASP at 0.3 mM, a concentration found in patients' sera [27], indicates that the two cell lines critically depend on the  $x_c^-$  transporter for sustenance of their intracellular GSH levels.

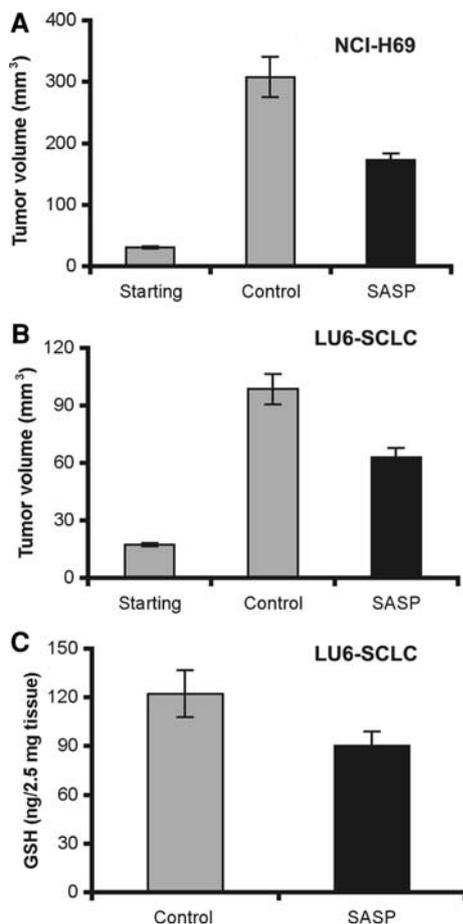
#### Effect of SASP on xenograft growth and GSH content

Mice carrying actively growing NCI-H69 and LU6-SCLC subrenal capsule xenografts were treated with SASP (250 mg/kg body weight; i.p.; b.i.d.) for 14 and 7 days, respectively. The treatment durations were well within the period of active tumor growth (data not shown). SASP markedly inhibited the increase in tumor size (as measured



**Fig. 2** SASP-induced reduction of proliferation and GSH content of NCI-H69 and NCI-H82 SCLC cell cultures: prevention by 2-ME. **a** Cultures of the cells in MEM supplemented with FBS (10%) and antibiotics were incubated for 72 h in the absence of SASP (control) and with SASP at the concentrations indicated. **b** In parallel cultures, incubation was carried out with added 2-ME (66  $\mu$ M) to allow cellular uptake of cystine via a route circumventing the  $x_c^-$  transporter (see text). In both cases, cell viability was assayed using a trypan blue exclusion assay. Data are expressed as number of viable cells relative to controls, means  $\pm$  SD, and are representative of results from two experiments. **c** Cells were incubated for 24 h with 0.1 and 0.3 mM SASP in the presence and absence of 2-ME (66  $\mu$ M) and their total GSH contents and viability determined. Total GSH levels ( $\mu$ g/well normalized to the control) are expressed as means  $\pm$  SD and are representative of two experiments

with calipers) of both NCI-H69 and LU6-SCLC xenografts with growth inhibitions of 51 ( $P < 0.01$ ; Fig. 3a) and up to 56% ( $P < 0.01$ ; Fig. 3b), respectively. The treatments with SASP did not lead to major toxic side effects in the mouse hosts. In addition, the GSH content of LU6-SCLC xenografts was measured at the end of the treatment. As shown



**Fig. 3** Effect of SASP on growth of human SCLC xenografts and GSH content. Groups of NOD-SCID mice ( $n = 5$ ) carrying subrenal capsule xenografts of NCI-H69 SCLC cells or LU6-SCLC tissue (2 grafts/kidney) were treated for 14 and 7 days, respectively, with saline (controls) or SASP (250 mg/kg; i.p.; b.i.d.). Tumor volume (measured with calipers) was expressed in cubic millimeter; data presented as means  $\pm$  SEM are representative of two (NCI-H69) and three (LU6-SCLC) experiments. Total GSH levels were determined in LU6-SCLC xenograft tissue (day 8); data presented as means  $\pm$  SEM are representative of two independent determinations (triplicate). Effects of SASP on **a** NCI-H69 cell xenograft growth, **b** LU6-SCLC tissue xenograft growth, **c** total GSH levels in LU6-SCLC xenografts

in Fig. 3c, the GSH levels in the SASP-treated tumors were significantly lower (26%) than those in the control tumors ( $P = 0.03$ ).

#### Effect of SASP on LU6-SCLC xenograft growth and vascularity determined via a stereologic approach

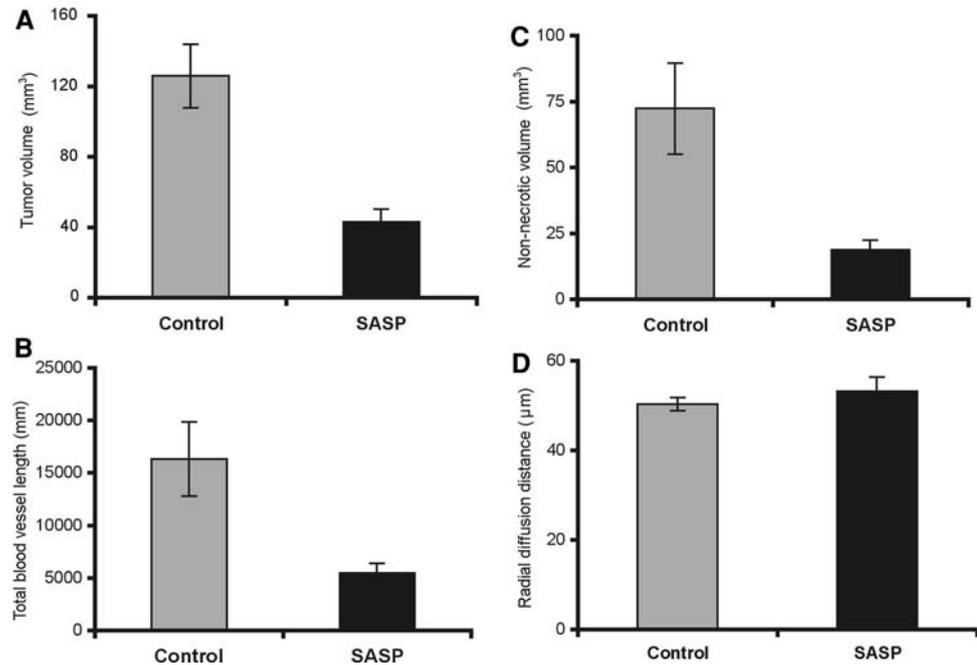
During a 7-day treatment with SASP the growth of the LU6-SCLC tumor tissue line was markedly inhibited ( $\sim 70\%$ ) when compared to the control ( $P < 0.01$ ) (Fig. 4a). The volume of tissue composed of non-necrotic tumor cells was also substantially lower ( $P < 0.01$ ) in the SASP-treated group (Fig. 4b). The proportion of tissue made up of various

categories of necrotic cells was not significantly altered with SASP treatment, although there was a tentative increase in the case of ‘severe necrotic’ cells (Table 1). SASP had no effect on the number of blood vessels per unit area ( $N_v$ ) or their length density ( $L_v$ ) (Table 1). Whereas the total length of blood vessels in the tumor tissue was significantly smaller in the SASP-treated group (Fig. 4c), the mean radial diffusion distance was not altered (Fig. 4d). This would suggest that the vascular bed is appropriate for the size of tissue mass.

#### Discussion

Glutathione, a major cellular scavenger of free radicals, is considered essential for protection of cells from oxidative stress. Such stress is particularly generated in cancer cells due to their relatively high metabolism. As a rate-limiting GSH precursor, the amino acid cystine/cysteine is of critical importance for maintenance of intracellular levels of GSH and hence has a vital role in the protection of cellular growth and viability [11, 13]. The present study has demonstrated that the human SCLC cell lines, NCI-H69 and NCI-H82, cannot synthesize sufficient amounts of cysteine and require additional amounts of the amino acid from their micro-environment for GSH synthesis and growth (Figs. 1a, 2). Uptake by these lung cancer cells of cystine, the main form of the amino acid in culture medium [11, 28], was primarily mediated by the  $x_c^-$  cystine transporter. Thus incubation of the cells with MSG, a highly specific  $x_c^-$  inhibitor [11], led to substantial growth inhibition (Fig. 1c), which was similar to that obtained by omitting cystine from the culture medium (Fig. 1a). Similarly, SASP, a potent but less specific  $x_c^-$  inhibitor used at relatively low, patient-tolerated levels ( $\sim 0.3$  mM) [27], induced marked growth inhibition which was coupled to extensive intracellular GSH depletion ( $>70\%$  at 24 h) (Fig. 2). Importantly, the growth arrests and GSH depletion could be largely prevented via inclusion in the culture medium of cystine uptake-enhancing 2-ME (66  $\mu$ M; Figs. 1c, 2b, c), a compound that allows shuttling cystine into cells (via the L-transport system) while circumventing inhibition of the  $x_c^-$  transporter [26]. It may be noted that 2-ME at a concentration of 50–100  $\mu$ M does not affect the molecular structure of SASP, as previously shown via High Performance Liquid Chromatography of SASP samples treated with 2-ME for extended periods (A.R.B. et al., unpublished observations). This indicates that the SASP-induced reduction of GSH levels and growth was primarily based on inhibition of cystine uptake leading to cystine starvation, as previously found for a variety of cancer cell lines [5, 7, 15]. There was a negative correlation between the SASP sensitivity of the cell lines and their degree of  $x_c^-$

**Fig. 4** Stereologic analysis of the effect of SASP on LU6-SCLC tissue xenografts (see Fig. 3b). Effects of SASP on **a** tumor volume (mean  $\pm$  SEM) ( $P < 0.01$ ). **b** Volume (mean  $\pm$  SEM) of non-necrotic tissue ( $P < 0.01$ ). **c** Total blood vessel length (mean  $\pm$  SEM) ( $P < 0.01$ ). **d** Radial diffusion distance (mean  $\pm$  SEM) (not significant)



transporter expression as measured by RT-PCR of *xCT*-mRNA. Thus the NCI-H82 cells, showing relatively low *xCT* expression (Fig. 1b), exhibited higher sensitivity to SASP than the NCI-H69 cells in the range 0.1–0.2 mM SASP (Fig. 2a). This would be consistent with action of SASP based on specific targeting of the  $x_c^-$  transporter. Taken together, the results indicate a critical growth requirement of the lung cancer cells for extracellular cystine/cysteine.

Although MSG inhibits the  $x_c^-$  transporter more specifically than SASP, its neurotoxicity precludes its use in higher organisms, in contrast to SASP which is a relatively non-toxic, FDA-approved drug [5, 29]. Growth of both NCI-H69 and LU6-SCLC xenografts in NOD-SCID mice was markedly inhibited by SASP as shown by tumor volume measurements, with evidence of reduced GSH levels in SASP-treated LU6-SCLC xenografts (Fig. 3a–c) and without major side effects to the mouse hosts. Stereologic analysis not only confirmed that SASP substantially restricted the growth of the LU6-SCLC tissue in vivo (Fig. 4a, b), but also indicated that the corresponding vascular bed was proportionate for the size of tumor, in particular since the mean radial diffusion distance was not altered (Fig. 4d), leaving open the question as to whether the reduced tumor growth was the result of inhibited vascular growth or vice versa. At present there is no explanation for the lack of difference between SASP-treated and control tumor tissue in the proportion of tissue made up of various categories of necrotic cells (Table 1). However, in this context it is of interest that recent observations in our laboratory have shown that SASP can

**Table 1** Stereological parameters (mean  $\pm$  SEM) of patient-derived subrenal capsule LU6-SCLC xenografts in NOD-SCID mice that were not significantly affected by SASP treatment (see Fig. 3b)

Parameters	Control	SASP	<i>P</i> value
Vv (non-necrotic): total	0.54 $\pm$ 0.07	0.43 $\pm$ 0.05	NS
Vv (mild necrosis): total	0.30 $\pm$ 0.02	0.33 $\pm$ 0.03	NS
Vv (severe necrosis): total	0.16 $\pm$ 0.06	0.24 $\pm$ 0.05	NS
Na: number of vessels per unit area	61.9 $\pm$ 6.4	62.4 $\pm$ 7.8	NS

Vv volume fraction, Na number per unit area, NS not statistically significant

promote autophagy of target cells (unpublished observations).

In analyzing the SASP-induced suppression of the SCLC growth in the animals, one has to take into account that in vivo, as distinct from in vitro, the cancer cells were not only exposed to cystine, but also to cysteine which can be readily taken up by cells via e.g., the universally expressed ASC transport system [11]. Although cysteine levels in the circulation are in general very low [28], cells can apparently acquire the necessary amounts of cysteine for their functions from transient increases in cysteine levels in their micro-environment, namely as cysteine is secreted by somatic cells in their vicinity—a process that involves the  $x_c^-$  transporter [11, 12]. Thus somatic cells such as fibroblasts, activated macrophages, and dendritic cells are known to take up extracellular cystine via the  $x_c^-$  transporter, reduce it intracellularly to cysteine and secrete cysteine into the extracellular compartment [11, 12].

Cancer cells which depend on extracellular cystine/cysteine but do not express a plasma membrane cystine transporter (such as Nb2–11 lymphoma cells [4, 5]), are particularly dependent on supply of cysteine, and their growth can be readily arrested by  $x_c^-$  inhibition interfering with the secretion of cysteine by neighboring somatic cells [10, 11]. In this context it may be noted that there is at present no information on expression of the  $x_c^-$  transporter by human lung cancer cells in vivo. However, as indicated above, such cells, if dependent on extracellular cystine/cysteine, would not need to express the transporter in vivo since they can obtain the amino acid in the form of cysteine as supplied by somatic cells. Without expressing a cystine transporter they would still respond to  $x_c^-$  inhibitors, although indirectly, due to reduction of the cysteine supply [10–12].

With regard to the present study it appears that the SASP-induced growth inhibition of the SCLC xenografts is to a major extent based on inhibition of the  $x_c^-$  transporter leading to reduced GSH levels, as indicated by the SASP-induced reduction of intracellular GSH levels in the SCLC cells, both in vitro and in vivo. A similar in vivo tumor growth inhibition coupled to reduction in GSH contents has been reported by other researchers for glioma xenografts treated with SASP [30]. However, the SASP-induced SCLC growth inhibition may involve additional mechanisms. Thus SASP has been reported to interfere with cytokine production by macrophages, and intra-peritoneal administration of SASP to mice can lead to apoptosis of peritoneal macrophages [31] and hence to reduction in the number of growth-supporting macrophages in the micro-environment of the tumor cells [10, 11, 32]. Further studies in this area appear therefore warranted.

Treatment with SASP has also been shown to lead to sensitisation of cells to drug treatment. For example, SASP has been reported to suppress in vitro drug resistance of certain lung adenocarcinoma cells [33], and breast cancer cells [15], rendering them more sensitive to doxorubicin. The sensitisation property of SASP has been attributed to its inhibition of NF $\kappa$ B activation [34], but probably also stems from SASP-induced GSH depletion [15], a condition that encompasses lack of defense against drug-induced oxidative stress [13, 35–37]. SASP is relatively non-toxic and hence could be useful in combination with conventional drugs to lower drug resistance and reduce toxic side-effects.

The present study suggests that small-cell lung cancers may have a growth requirement for extracellular cystine/cysteine. In that case the  $x_c^-$  cystine/glutamate antiporter could provide a new target for therapy of the disease based on glutathione depletion. The finding that the human LU6-SCLC tissue xenograft model responded markedly to treatment with sulfasalazine, an FDA-approved drug with

potent  $x_c^-$ -inhibitory activity, indicates that this drug may be useful for clinical therapy of SCLC, in particular since the LU6-SCLC line was derived from a cancer resistant to multiple chemotherapy drugs. Since sulfasalazine can lead to cell sensitisation, it could be especially valuable as a component of combination chemotherapy aimed at reducing resistance to conventional anticancer drugs.

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