S-allylcysteine, a water-soluble garlic derivative, suppresses the growth of a human androgen-independent prostate cancer xenograft, CWR22R, under in vivo conditions

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OBJECTIVE
To evaluate the effect of S-allylcysteine (SAC) on CWR22R, a human androgen-independent (AI) prostate cancer xenograft, in nude mice. Despite extensive research worldwide there is no effective way to control the growth of prostate cancer, and we previously reported that SAC and S-allylmercaptocysteine (SAMC), two water-soluble derivatives of garlic, inhibit cancer cell invasion through restoration of E-cadherin expression in vitro.

MATERIALS AND METHODS
The effects of SAC on tumour cell proliferation markers such as Ki-67 and proliferating cell nuclear antigen, and apoptotic regulators including Bcl-2 and cleaved caspase-3, were assessed by immunohistochemical staining. The inhibitory effects of SAC on prostate cancer invasion was examined by immunoreactivity of E-cadherin and its binding proteins α, β and γ-catenins. The serum prostate-specific antigen (PSA) level at three different times (initiation, middle and end of treatment) and toxicity of SAC on several organs after treatment were assessed.

RESULTS
Treatment with SAC resulted in inhibition of the growth of CWR22R, with no detectable toxic effect on nude mice. The SAC-induced growth reduction was correlated with a concurrent reduction in serum PSA level and proliferation rate of xenografts, together with an inhibition of invasion through the restoration of E-cadherin and γ-catenin expression. Furthermore, the apoptotic rate of SAC-treated tumours increased together with a decrease in Bcl-2 and increase in cleaved caspase-3.

CONCLUSION
These results suggest that this garlic-derived compound might be a potential therapeutic agent for suppressing AI prostate cancer.

KEYWORDS
s-allylcysteine, prostate cancer, apoptosis, proliferation, invasion

INTRODUCTION
Prostate cancer is one of the most prevalent invasive malignancies and the second leading cause of cancer-related death in men in Western countries [1,2]. By contrast, the incidence of prostate cancer in China, including Hong Kong, has been relatively low, but has rapidly increased in recent years, although it is still much lower than in the West [3]. Localized early-stage androgen-dependent prostate cancer is manageable with systemic therapy, including surgery, radiotherapy and androgen-depletion therapy. However, once the disease progresses to the androgen-independent (AI) stage, especially metastatic stages, there are no effective ways to control tumour growth. Therefore, the development of novel treatment approaches is needed to prolong the survival and improve the quality of life in these late-stage patients.

In the past several decades, natural dietary agents such as those from garlic (Allium sativum) have had much attention because they have chemopreventive and therapeutic potential against various cancers, e.g. of the digestive tract and prostate [4–7]. Garlic contains high levels of organosulphur compounds, the main anticancer constituents, which can be primarily divided into two groups. One group is lipid-soluble, including diallyl sulphide, diallyl disulphide (DADS) and diallyl trisulphide (DATS). The other group is water-soluble, e.g. S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC). In aged garlic extract (AGE) the contents of SAC and SAMC are high and with few lipid-soluble compounds. AGE is produced by immersion and extraction of sliced raw garlic in aqueous ethanol for ≥10 months at room temperature. During this process of ageing, odoriferous, resistant lipid-soluble compounds are changed naturally into more stable and bioavailable water-soluble compounds [8–10].

Also as a common dietary component, garlic and its derivative constituents were suggested to be effective chemopreventive agents, based mainly on epidemiological studies and carcinogenic processes in several experimental animal models. For example, a population-based case-control study conducted in China, using 238 patients with confirmed prostate cancer and 471 normal male controls, reported that men with a high intake of garlic (>2.14 g/day) had a significantly lower risk of prostate cancer than those with low or no garlic consumption [5]. An experimental carcinogenesis study showed that AGE inhibits colon carcinogenesis by suppressing cell proliferation in 1,2-dimethylhydrazine-induced colon cancer.
in rats [11]. These results indicate that garlic might have positive effects in the chemoprevention of certain human cancers.

To date the evidence for possible therapeutic effects of garlic and its derivative components mainly derives from in vitro studies on various cancer cell lines, including colon, lung, leukemia, skin, breast and prostate [4,7]. These preclincial studies provided strong evidence to indicate that garlic-derived organosulfur compounds, including the lipid-soluble compounds DADS and DATS, and water-soluble compounds SAC and SAMC, are highly effective in suppressing the proliferation of cancer cells in culture by inducing apoptosis or arresting cell cycle progression. For example, it was shown that DATS can suppress the proliferation of prostate cancer cell lines by inducing apoptosis through inhibiting the expression of Bcl-2 protein, and activating ERK1/2 and JNK pathways, as well as inactivating the Akt signalling axis [12,13]. In addition, DADS and SAMC inhibit colon cancer cell growth by interfering with its microtubule assembly, which triggers the JNK1 and caspase-3 signalling pathways, leading to apoptosis [14,15]. A recent study from our laboratory showed that SAC and SAMC inhibit the invasive ability of several cancer cell lines, including prostate cancer cells, by restoring E-cadherin expression, the down-regulation of which is one of the most common features of metastatic cancer [16]. All these studies suggest that in addition to their cancer-preventive effects, garlic and its derivatives might also have therapeutic effects against prostate cancer by modulating several pathways. However, more detailed in vivo studies are needed to establish the therapeutic potential of garlic derivatives such as SAC and SAMC against prostate cancer xenografts in animal experiments. Furthermore, the underlying mechanisms responsible for the effects of garlic derivatives also warrant further investigation to improve the strategy for treating prostate cancer.

The present study was designed to determine the anticancer effect of SAC on an AI human prostate cancer xenograft, CWR22R, in athymic nude mice, by monitoring its effect on tumour growth, serum PSA level and body weight, as well as its toxicity on several vital organs.

MATERIALS AND METHODS

SAC was generously provided by Wakunaga Pharmaceutical Co., Ltd. (Hiroshima, Japan) and was used in our previous studies [16]. A stock solution of SAC (1000 mg/16 mL) was prepared freshly in PBS according to the manufacturer’s suggestion.

For the CWR22R prostate cancer xenograft model, male BALB/c athymic nude mice (6–7 weeks old) were used. Mice were housed under standard conditions (20 ± 2 °C, 40–70% relative humidity, 12-h light/dark cycle) with food and water supplied ad libitum in a pathogen-free environment. CWR22R is widely accepted as an AI human prostate cancer xenograft, derived from CWR22 after castration of the host [17–19] (from Dr FL Chan, CUHK). CWR22R xenografts were maintained in castrated nude mice as described previously [17]. Briefly, mice were castrated at least 2 days before tumour implantation. An established tumour from a host mouse was minced into small fragments (to pass through an 18 G needle), and the tissues extensively washed with RPMI 1640 medium and then mixed with Matrigel (1 : 1 v/v, BD Bioscience, Bedford, MA, USA). About 0.1 mL of the tumour-Matrigel mixture was injected s.c. into each flank of castrated mice. All surgical operations were performed under aseptic conditions and the studies were conducted in accordance with the guidelines of the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong.

In all, 22 mice carrying palpable CWR22R tumours of similar sizes (about 1 week after transplantation) were selected and randomly divided into two equal groups. Each group was treated with SAC (1000 mg/kg, i.p. injection) or vehicle (PBS) daily for 7 weeks. The mice were weighed weekly during the study (all 22 mice). Blood samples (≈ 100 µL) were collected from the thigh vein of each mouse before starting drug treatment (i.e. 1 week after tumour inoculation), in the middle of treatment (i.e. 5 weeks after tumour inoculation) and a cardiac bleed was carried out at death (i.e. 8 weeks after tumour inoculation). Total PSA was detected using the 96-well enzyme immunoassay kit (CanAg Diagnostics AB, Gothenburg, Sweden). All samples, including all calibrators, controls and experimental samples, were assessed in duplicate and the PSA values were calculated from the calibration curve using the mean absorbance value of each sample. To determine the changes in liver function, serum alanine aminotransferase (ALT or SGPT) and aspartate aminotransferase (AST or SGOT), indicators of liver toxicity, were determined at the end of treatment (22 mice) using the automated Architect ci8200 integrated system (Abbott Diagnostics, Abbott Park, IL, USA).

The immunohistochemical analysis used a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Sections were deparaffinized in toluene and rehydrated to distilled water. Endogenous peroxidase activity was blocked by treating the specimens with 0.3% hydrogen peroxide in methanol for 30 min, followed by rehydration in Tris buffered saline (TBS) and incubation with normal mouse serum for 30 min to primary mouse monoclonal antibodies against proliferating cell nuclear antigen (PCNA; Ab-1, 1 : 2500; Oncogene Science, Manhasset, NY, USA), Ki-67 (clone MM1, 1 : 100; Novocastra Laboratories Ltd, Newcastle, UK), Bcl-2 (C-2, 1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin, α, β and γ-catenin (1 : 200; BD Transduction Laboratories, San Jose, CA, USA) and rabbit polyclonal antibody against cleaved caspase-3 (Asp175, 1 : 50; Cell Signalling Technology, Inc., Beverly, MA, USA). After several rinses in TBS, the sections were incubated with biotinylated anti-mouse IgG at a dilution of 1 : 200 for 45 min at room temperature, followed by peroxidase-conjugated avidin–biotin complex and 3,3′-diaminobenzidine (DAKO, Carpinteria, CA, USA). The sections were then counterstained with Mayer’s haematoxylin and analysed by standard light microscopy. To quantify the immunohistochemical reactivity, cells reacting positively with PCNA,
Ki-67 (nuclear) and cleaved caspase-3 (both cytoplasmic and perinuclear) were counted using a computer-based image-analysing system (Stereo Investigator, VT, USA) attached to a microscope at ×100 from five randomly selected fields per tumour, and the percentage of positive cells was calculated as: number of positive cells/total number of cells × 100. A semiquantitative method was used for scoring Bcl-2 immunoreactivity; briefly, 10 random microscopic fields per tumour (at ×400) from the control and SAC-treated groups were assessed and scored based on their staining intensity, ranging from grade 0 to II. Grade 0 (negative) was defined as no or marginal staining of <5% of cells, grade I as mild to moderate staining of 5–50% of cells, and grade II as moderate to intense staining comprising more than half the cells. All evaluations of reactivity and counting of the slides were carried out while unaware of sample origin, by two reviewers.

The results were analysed using the Mann–Whitney U-test and Student’s t-test, with P < 0.05 considered to indicate significant differences between samples; all quantitative data are presented as the mean ± (SEM).

RESULTS

The suppressive effect of SAC on cancer was investigated under in vivo conditions using the AI human prostate cancer xenograft CWR22R, which mimics the clinical events of human relapsed prostate cancer [17,20]. The gross size of representative tumours in the control group was much larger than in the SAC-treated group after 7 weeks of SAC treatment (Fig. 1A). In addition, daily treatment with SAC resulted in a reduction of 62.4% in the mean final tumour volume compared with the vehicle-treated control at the end of treatment (Fig. 1B). The effect was apparent as early as 4 weeks of treatment with SAC, and the differences became more obvious with longer treatment.

Earlier studies showed that the blood PSA level is related to tumour volume, and thus can be used as an index for tumour growth in this CWR22R model [21,22]. Therefore we measured the serum PSA level in both groups of mice at the three times stated; as shown in Fig. 1C, before treatment the PSA levels were very low and there was no difference between the groups of mice. As the treatment time increased, the serum levels of PSA were also
increased in both groups, but the increase was much slower in the SAC-treated than the control group at the middle and end of SAC treatment. Taken together, these results indicate that SAC can suppress the growth of the AI prostate cancer xenograft in nude mice and the effect correlates well with the reduction of serum PSA level.

The well-being of SAC-treated mice was assessed by monitoring the food and water intake and body weight; the toxicity was evaluated by examining the histological features of several vital organs (liver, lung and kidney) together with serum markers (i.e. ALT and AST) of liver function in both groups of mice. The data showed that i.p. injections with SAC caused no detectable toxicity in terms of food and water consumption (data not shown) or body weight (Fig. 2A). In addition, as shown in Fig. 2B,C, there were no obvious changes in indicators of liver toxicity and histological features in lung, liver and kidney in control and SAC-treated mice.

We then investigated the possible underlying mechanisms of prostate cancer xenograft inhibition induced by SAC in nude mice. The expression of two proliferation markers, PCNA and Ki-67, were examined to determine whether SAC had any effect on cancer cell proliferation. As shown in Fig. 3A, there was a significantly lower percentage of cancer cells with positive nuclear reactivity for Ki-67 in the SAC-treated group, at 37.1 (4.8)% than in the control mice, at 69.7 (4.5)%. The result was similar for PCNA (Fig. 3B), which showed that the percentage of cancer cells with positive nuclear PCNA reactivity changed from 67.4 (8.9)% in the control to 42.7 (5.7)% in the SAC-treated group. These results suggest that the suppressive effect of SAC on prostate cancer growth is correlated with a decreased rate of cell proliferation.

Because SAC was reported to induce apoptosis in prostate cancer cells, we determined the effect of SAC on caspase-3 and Bcl-2, both known to regulate and execute programmed cell death. The cleaved caspase-3 antibody is more sensitive and specific than caspase-3 for examining apoptosis in paraffin sections [23,24]. As shown by immunohistochemical staining, the percentage of cleaved caspase-3 positive cells was higher in SAC-treated mice than in the control group (Fig. 4A). We further investigated the expression of Bcl-2 to confirm the apoptotic promoting effect of SAC.
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DISCUSSION

The present study was designed to evaluate the in vivo anticancer potential of SAC in castrated nude mice, using the human AI prostate cancer xenograft CWR22R. The results show clearly that SAC can suppress the growth of the CWR22R xenograft. The effects were correlated closely with a significant decrease in tumour cell proliferation and an increase in apoptosis. Furthermore, the study also showed that SAC can restore E-cadherin function under in vivo conditions, which reaffirms our previous in vitro findings on cancer cell lines [16].

CWR22R is known to secrete PSA and thus mimics closely the clinical scenario of relapsed prostate cancer [17–19]. It has been used widely for evaluating the effectiveness of drugs and their underlying molecular mechanisms under in vivo conditions [20,22]. Despite its shortcomings, the serum PSA level is still considered as the best marker for diagnosis and the effectiveness of treatment for prostate cancer in clinical practice. Usually the expression of PSA correlates well with various treatment strategies at the androgen-dependent stage, as the expression of PSA is mainly induced by androgens and regulated by the androgen receptor (AR) at the transcriptional level. However, it is also well known that PSA levels often increase in the AI stage; this increase in serum PSA level in relapsed and metastatic tumour is thought to be due to PSA gene re-expression mediated by AI AR activation [27]. This could be due to AR overexpression resulting from amplification of the AR gene, and thus could be activated by low levels of androgens from other than testicular sources. In the present study treatment with SAC caused a significant reduction in the volume of CWR22R tumours, together with a decrease in serum PSA levels (Fig. 1). The results indicated that the serum PSA level correlated well with tumour size and thus could be used as an additional index of the efficacy of SAC treatment on CWR22R besides the volume of the implant, which might include necrotic tissues.

The present results also showed that SAC treatment, at the dosage used, caused no apparent changes in liver function, histology of vital organs or body weight of the nude mice (Fig. 2). The results are also consistent with several earlier studies which showed that SAC was preventative in the process of

FIG. 4. Effect of SAC on apoptosis of CWR22R xenografts; control and SAC-treated tumours were stained immunohistochemically using antibodies against cleaved caspase-3 and Bcl-2. (A) Representative photomicrographs of immunohistochemical staining against cleaved caspase-3 in the control (panel 1) and SAC-treated (panel 2) groups, and quantification of caspase-3-positive cells in both groups (right panel). (B) Photomicrographs of representative tumours with Bcl-2 staining intensity of grades II and I in the control (panels 1, 2) and grades 1 and 0 in the SAC-treated tumours (panel 3, 4) and illustrative results of semiquantitative values of Bcl-2 staining in both groups (right panel). (A) Representative photomicrographs of cleaved caspase-3 expression and reduced Bcl-2 expression.

SAC; most tumour tissues from control mice (95.7%) had strong to moderate Bcl-2 staining intensities, whereas most cells in SAC-treated tumours (89%) had only weak to undetectable levels (Fig. 4B). These results indicate that the tumour-suppressive effect of SAC might be mediated through an increase in apoptosis, accompanied by activation of caspase-3 and a concurrent down-regulation of Bcl-2.

Our recent in vitro study showed that SAC and SAMC cause inhibition of invasion by restoring E-cadherin expression in prostate cancer cells; thus we used immunohistochemistry for E-cadherin and its cytoplasmic binding proteins α, β and γ-catenin proteins in the control and SAC-treated tumours. There was greater expression of E-cadherin and γ-catenin proteins in the SAC-treated tumours than in the controls (Fig. 5), but there were no changes in both α- and β-catenin expression in tumour tissues after treatment with SAC (data not shown). In addition, there was membrane localization of the E-cadherin and γ-catenin proteins, which have been generally accepted to indicate an activation of both proteins [25,26], in SAC-treated tumour cells. By contrast, there was only weak cytoplasmic positive E-cadherin and γ-catenin staining in the untreated controls. These results are in full agreement with our findings in vitro and imply that SAC treatment might also be able to inhibit the invasive ability of CWR22R by restoring the function of E-cadherin and γ-catenin, thus further suppressing cancer metastasis.

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chemically induced carcinogenesis, while showing no apparent sign of toxicity in vivo [28–30]. Interestingly, one study showed that SAC might even attenuate the toxicity of doxorubicin treatment in cancer chemotherapy [31]. These lines of evidence suggest that SAC might be a potential candidate for patients with late-stage prostate cancer to delay tumour progression. Currently, patients with late-stage prostate cancer have limited drug options and are often less tolerant of available therapeutic drugs. SAC, in view of its effectiveness in restricting the growth of tumour, and low toxicity as shown in the present study, might be a potential option.

The dosage used (1 g/kg body weight) might be considered high if directly translated to humans in clinical trials, although this dosage is equivalent to ∼14% of the median lethal dose as reported by earlier investigators [30]. More importantly, as shown in the present results, at this dosage there was no obvious detectable toxicity to mice, which is supported by the present liver-function study. Although it would be difficult to use this dosage directly in patients at this stage as a sole treatment approach, it might not be unachievable. For example, 70 g of SAC could be dissolved in 1 L of physiological saline and then administered daily to patients by i.v. drip, or a lower dose could be used as an adjuvant combined with other treatment strategies. The risk/toxicity should be assessed carefully before application to patients.

We investigated the mechanisms involved in the tumour-suppressive effect of SAC; the results showed that the decreased tumour proliferation rate (Fig. 3) and increased apoptosis of tumour cells (Fig. 4) might be responsible for the growth reduction of CWR22R tumour xenografts by SAC (Fig. 1). The antiproliferative effect of SAC was evident from the results showing that the reactivity of doxorubicin in vitro report and others which showed that SAC can inhibit the proliferation of cancer cell lines [4,7,16]. In addition, there was also a greater expression of cleaved caspase-3 and decreased Bcl-2 reactivity in the SAC-treated tumours in the present study. Recently regulatory molecules of apoptosis have been used as targets for some dietary bioactive agents, as chemopreventive and potential therapeutics of cancer [32]. Among those molecules, greater expression of Bcl-2 detected in locally advanced prostate cancer correlated with higher stage and grade in AI cell lines than androgen-dependent cells. Apoptosis is a multistep process involving both intrinsic and extrinsic pathways with a balance of pro-apoptotic (e.g. caspase-3) and anti-apoptotic protein, including Bcl-2. Both pathways are correlated with activation of caspsases, especially caspase-3, which is one of the several targets for apoptosis. Bcl-2 is generally accepted as an anti-apoptotic gene and an integral regulator of the intrinsic mitochondrial pathway involved in preventing the release of caspsases, including caspase-3, which activates apoptosis by stimulating cleaved caspase-3 activity [32,33]. In this context, importantly the present data are in good agreement with results reported by others under in vitro conditions [34]. Therefore, our findings suggest that induced activation of the apoptotic pathway might be another mechanism responsible for the inhibition of CWR22R xenograft by SAC in vivo.

The down-regulation of E-cadherin and its cytoplasmic binding proteins, catenins, is associated with high-grade tumours and a poor prognosis in prostate cancer, indicating their role in progression of the cancer [35,36]. E-cadherin, together with α and γ-catenin, one of two distinct adhesion complexes, is essential for normal E-cadherin function. Previously, an in vitro study in our laboratory showed that SAC and SAMC can inhibit the invasive ability of prostate cancer cells by restoring the E-cadherin–γ-catenin interaction [16]. In the present study there was also greater expression of E-cadherin and γ-catenin proteins in the SAC-treated CWR22R xenografts than in controls (Fig. 5), which is consistent with our findings in prostate cancer cell lines [16]. That both E-cadherin reactivity and membrane localization were greater in the SAC-treated human AI prostate cancer xenografts further confirms that the SAC-induced E-cadherin activation, as shown earlier under in vitro conditions [16], also occurs under in vivo conditions.

Taken together, the current study highlights the potential therapeutic effect of a garlic derivative, SAC, in AI prostate cancer. The in vivo anticancer effect of SAC might be mediated through inhibition of proliferation and invasion, while promoting apoptosis.

**FIG. 5.** The effect of SAC on E-cadherin and γ-catenin expression in CWR22R tumour xenografts, assessed immunohistochemically using antibodies against E-cadherin and γ-catenin. The representative images of controls for E-cadherin and γ-catenin are shown in panels 1 and 3, respectively, while those of SAC-treated mice are shown in panels 2 and 4, respectively. ×400. SAC treatment lead to increased membranous expression of E-cadherin and γ-catenin.
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CONFLICT OF INTEREST

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Abbreviations: AI, androgen-independent; AGE, aged garlic extract; SAC, S-allylcysteine; SAMC, S-allylmercaptocysteine; DADS, diallyl disulphide; DATS, diallyl trisulphide; PCNA, proliferation cell nuclear antigen; TBS, Tris buffered saline; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AR, androgen receptor.