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Effects of glycyrrhetinic acid and liquorice extract on cell proliferation and prostate-specific antigen secretion in LNCaP prostate cancer cells

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Abstract

Glycyrrhetinic acid (GA) is the active metabolite of glycyrrhizic acid, one of the components of liquorice extract. It has been shown to possess anti-inflammatory activity and to inhibit hepatic tumour growth. In this preliminary study, we have shown that GA could significantly reduce the rate of proliferation of LNCaP androgen dependent prostate cancer cells, whereas it had no effect on proliferation of PC3 and DU145 androgen-independent prostate cancer cells. Additionally, GA could significantly reduce the production of prostate-specific antigen by LNCaP cells maintained in-vitro. This study provides a sound platform for further investigation.

Introduction

The prostate undergoes significant growth at puberty when luteinising hormone from the anterior pituitary gland stimulates the testes to produce testosterone. 5α-Reductase then converts testosterone to dihydrotestosterone, the androgen that acts on prostatic epithelial cells (Kumar et al 2005). Androgens continue to be essential for normal growth and development of the prostate throughout life. It is accepted that prostate cancer is a complex disease with multifactorial aetiology; however androgenic hormones are obligatory survival factors for epithelial cells of the prostate gland.

Prostate-specific antigen (PSA) is an enzyme produced by normal and cancerous prostate cells but levels in the systemic circulation can increase significantly due to the development of prostate cancer and change in gland architecture (Oleyourryk & Messing 2001). Controversy exists over the role of androgen in the production of PSA. Sun et al (2001) and Jia et al (2003) stated that androgen stimulated the production of PSA, however Isom-Batz et al (2005) reported no evidence of an interaction between androgen and PSA. The production of PSA by cultured prostate cancer cells has been shown to be androgen responsive, with dihydrotestosterone evoking a marked increase in PSA secretion (Pecher et al 2004). Sadar et al (1999) reported that serum PSA and mRNA levels were down-regulated when androgen was withdrawn and up-regulated when it was replaced. PSA may play a role in prostate cancer progression through the activation of transforming growth factor-β (Killian et al 1993), modulation of cell adhesion (Killian et al 1993; Romanov et al 2004) and degradation of extracellular matrix (ECM) glycoproteins and modulation of invasion (Weber et al 1995; Lilja et al 2000). Many metastatic tumours continue to produce PSA, suggesting that it is beneficial to ongoing tumour cell growth and survival (Denmeade et al 2003).

Most human prostate cancer cells are initially responsive to androgen withdrawal as the cells grow in an androgen-dependent epithelium and initially are androgen sensitive (Minelli et al 2006). Conventional treatment options for androgen-dependent tumours, such as androgen ablation, are often associated with negative side effects, which can decrease quality of life to the extent that treatment is not beneficial. Recently there has been much interest into studying new compounds that may be used as an alternative or as an adjunct to improve the tolerability and efficacy of these aggressive treatments (Sternberg et al 2007). Prostate cancer is an ideal candidate for chemoprevention by natural products because it is typically diagnosed in men over 50 years of age, and so a delay in disease progression

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achieved through nutritional intervention could significantly impact the quality of life of these patients (Malik & Mukhtar 2006).

Liquorice (or licorice) (*Glycyrrhizia glabra*) is a root crop which grows wild in Southern Europe and Asia and has been used for centuries, not only as a flavouring and sweetener but also as a medicinal product (Armanini et al 2002). One of the main components of liquorice is glycyrrhizic acid (GL) which is metabolized in-vivo to the active aglycone glycyrrhetinic acid (GA), via intestinal bacterial glucuronidase activity (Akao et al 1994; Armanini et al 2003) (Figure 1).

GA can modulate the activity of a number of cellular enzymes in the normal prostate and testes. It has been shown to inhibit the activity of 17,20 lyase and 17β-hydroxysteroid dehydrogenase, which catalyse the conversion of 17-hydroxyprogesterone into androstenedione and androstenedione to testosterone, respectively (Yaginuma et al 1982; Sakamoto & Wakabayashi 1988; Takeuchi et al 1991). Additionally Latif et al (1990) showed that GA was a potent inhibitor of cytosolic 5α-reductase. Oral administration of GA decreased in-vivo basal testosterone production in Leydig cells, although it did not change the associated cAMP or progesterone levels (Sakamoto & Wakabayashi 1988).

Abe et al (1987) were the first to demonstrate that GA could also modulate the proliferation of cancer cells. They showed that it could inhibit the growth of B16 melanoma cells by blocking the cell cycle and stimulating melanogenesis. More recently GA was shown to induce G1 arrest and apoptosis in HepG2 human hepatocellular carcinoma cells in-vitro (Satomi et al 2005), with synthetic derivatives of GA having increased antiproliferative and apoptotic effects in human HL-60 leukaemia cells (Liu et al 2007). Recently, novel conjugates of GA with dehydrozingerone and paclitaxel have been tested as cytotoxic agents against a range of cancer cell lines maintained in-vitro (Nakagawa-Goto et al 2007; Tatsuzaki et al 2007).

In this preliminary study, we have shown that a crude liquorice extract and pure GA inhibited the proliferation of LNCaP androgen-dependent cells, whereas they had no effect on the growth of the PC3 and DU145 androgen-independent cell lines. Additionally, both compounds significantly down-regulated the production of PSA by LNCaP cells.

Materials and Methods

Cell culture

The human prostate cancer cell lines LNCaP, PC3 and DU145 were obtained from the American Type Culture Collection (Manassas, USA). They were maintained in RPMI 1640 medium (Invitrogen, UK) containing 10% fetal calf serum, at 37°C with 5% CO_2.

Liquorice and GA

Liquorice root capsules (Holland & Barrett) contained 420 mg powdered liquorice. Stock solutions were prepared by dissolving the contents of one capsule in 1 mL 70% ethanol. Samples were filtered to remove any remaining particulate matter. For in-vitro use, this stock solution was diluted in RPMI 1640 medium.

GA stock solutions (Aldrich, UK), prepared by dissolving in dimethyl sulfoxide (DMSO), were diluted in RPMI 1640 medium before in-vitro use.

Cell proliferation assay

Modified methods of those described by Jacob et al (1999) and Kuninaka et al (2000) were used. Essentially, cells were plated onto a 96-well plate (0.5×10^4 cells/well) and allowed to adhere overnight. Various concentrations of either liquorice or GA (0.1 μg mL^{-1}–20 mg mL^{-1}) were added to the cells, and the cells were allowed to grow for a further 48 h. MTT 0.4% (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (Sigma, UK) in DMSO 10 μL/well was added for a further 2 h to allow uptake of MTT by the cells. MTT is converted to purple formazan crystals by living cells, which is solubilized by the addition of 200 μL DMSO/well.

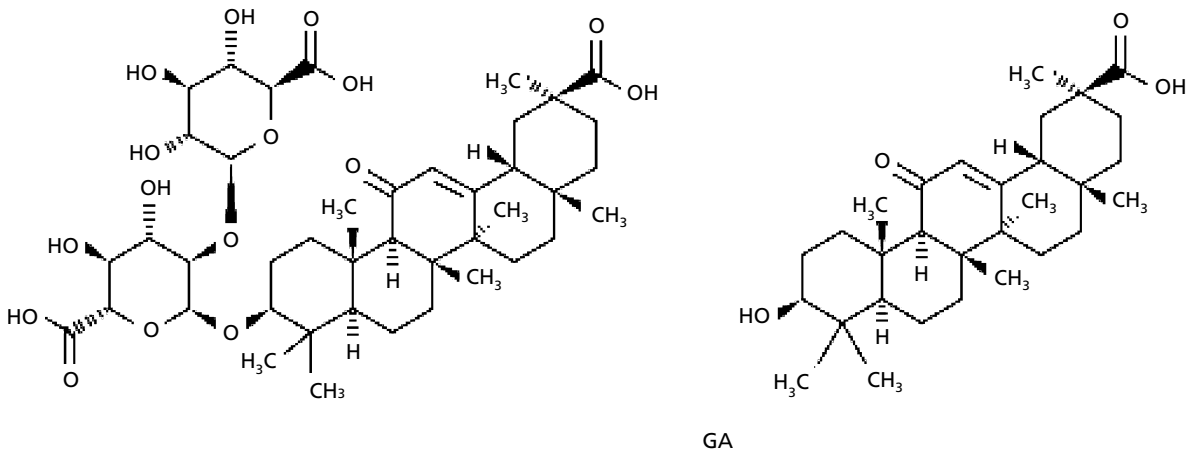

Figure 1 Structures of glycyrrhizic acid (GL) and glycyrrhetinic acid (GA).

Intensity of the colour was measured on a Tecan Spectrathermo spectrophotometer at 620 nm.

PSA measurement

A PSA ELISA kit (Goldenbridge International Inc., USA) was used to quantify the amount of PSA secreted by LNCaP cells; PC3 and DU145 cells do not produce PSA. Cells were grown in a 24-well plate at a concentration of 1×10^5 cells/well in the presence of either liquorice or GA for 24 h at 37°C. Supernatants were removed, centrifuged to pellet any cellular debris and stored at −70°C until required. The PSA ELISA was carried out according to the manufacturer's instructions. Briefly, samples and standards were incubated on the antibody-coated plate for 60 min. After washing to remove unbound antigen, monoclonal anti-PSA antibody was added for a further 60 min before addition of the 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance was read at 405 nm and the concentration of PSA was proportional to the optical density obtained.

Statistical analysis

Samples were analysed using the Kruskal–Wallis test. Individual differences between the various treatments were statistically analysed using Dunn's test. In all cases $P < 0.05$ denoted significance.

Results

Effect of liquorice extract and GA on proliferation of prostate cancer cell lines

To examine the antiproliferative effects of liquorice and GA, prostate cancer cell lines (LNCaP, PC3 and DU145) were cultured in the presence of various concentrations of either compound ($0.1\ \mu g\,mL^{-1}$–$20\ mg\,mL^{-1}$, 48 h). Liquorice and GA effectively inhibited the growth of LNCaP cells with an ED50 of 10.3 and $0.5\ mg\,mL^{-1}$, respectively (Table 1). Both compounds had little effect on the growth of PC3 and DU145 cells (Figure 2A and B).

Effect of liquorice extract and GA on the levels of secreted PSA

Under normal growth conditions, LNCaP cells secrete PSA into the medium at a concentration of approximately $27\ ng\,mL^{-1}$.

Table 1 ED50 values ($mg\,mL^{-1}$) for liquorice extract and GA with LNCaP, PC3 and DU145 prostate cancer cell lines

Treatment	Cell line		
	LNCaP	PC3	DU145
Liquorice extract	10.3	NA	NA
GA	0.5	NA	NA

NA, not applicable.

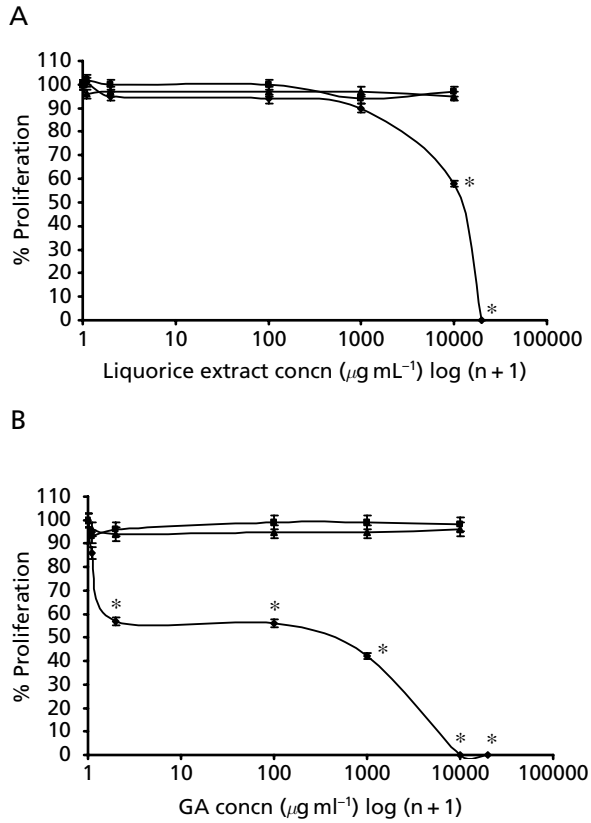

Figure 2 Semi-log graphs demonstrating the effect of liquorice extract and GA on the proliferation of prostate cancer cell lines determined by MTT assay. LNCaP, PC3 and DU145 cells were cultured with various concentrations of either liquorice extract (A) or GA (B) ($0.1\ \mu g\,mL^{-1}$–$20\ mg\,mL^{-1}$). After 48 h cells were treated with MTT for 2 h at 37°C and MTT activity measured. Results represent the mean ± s.d. of three experiments performed in triplicate. ♦ LNCaP cells; ■ PC3 cells; ▲ DU145 cells. *$P < 0.05$.

Liquorice extract (10 and $20\ mg\,mL^{-1}$, 24 h) down-regulated the secretion of PSA by 59.3% and 81.1%, respectively (Figure 3A). GA (1 and $10\ mg\,mL^{-1}$, 24 h) down-regulated the secretion of PSA by 39.8% and 63.7%, respectively (Figure 3B).

Discussion

Published research on the effects of liquorice and its constituents on prostate cancer cell growth is limited. PC SPES, a Chinese mixture of eight herbs containing liquorice, has been studied in greater detail. PC SPES is a complex mixture of phytochemicals and a number of the individual herbs are known to possess biological activity. It has been used as an alternative treatment for prostate cancer and the use of PC SPES in patients with prostate cancer can cause a reduction in the serum levels of PSA (Ikezoe et al 2003).

PC SPES can inhibit the proliferation of prostate cancer cells both in-vitro and in-vivo with induction of apoptosis being associated with down-regulation of bcl-2 (De la Taille et al 2000; Kubota et al 2000). Saw Palmetto, an isolate from

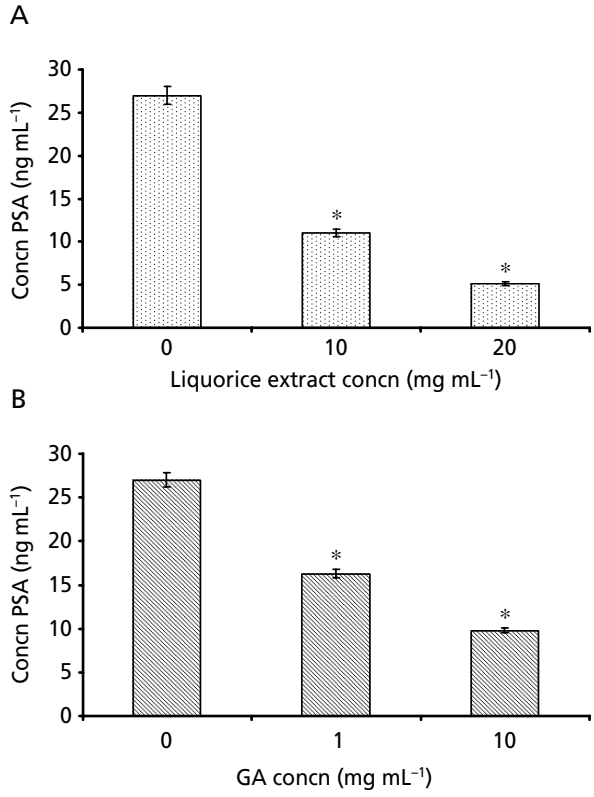

Figure 3 Effect of liquorice extract and GA on the secretion of PSA by LNCaP prostate cancer cells determined by PSA ELISA. LNCaP cells were grown in the presence of either liquorice extract (A) or GA (B) for 24 h after which time supernatants were collected and analysed in a PSA ELISA. Results represent the mean ± s.d. of three experiments performed in triplicate. $*P < 0.05$.

PC SPES, induced growth arrest of LNCaP androgen-dependent prostate cancer cells maintained in-vitro in a time- and dose-dependent manner via inhibition of STAT3 signalling (Yang et al 2007). It also down-regulated the levels of both PSA and androgen receptor in LNCaP cells suggesting that Saw Palmetto was at least one of the active components of PC SPES.

Isoliquiritigenin, one of the constituents of liquorice, inhibited the proliferation of LNCaP cells in a dose- and time-dependent manner due to the induction of S and G2/M phase arrest without initiating apoptosis (Kanazawa et al 2003). Conversely isoliquiritigenin induced apoptosis in gastric cancer MGC-803 cells (Ma et al 2001) and therefore biological effects may be cell-type dependent. Isoliquiritigenin differs in chemical structure from GA, being a flavonoid rather than a triterpenoid, and therefore GA may differ in its mechanism of action in LNCaP cells. The testing of other flavonoids present in liquorice may prove useful as they may also contribute to the activity of the total plant extract.

Permixon, another constituent of PC SPES, has been shown to possess anti-androgenic activity (Stenger et al 1982) by inhibiting the binding of androgen to the cytosolic androgen receptor in rat prostatic tissue (Carilla et al 1984) and human foreskin fibroblasts (Sultan et al 1984). Whilst it affects the rate of proliferation of androgen-dependent LNCaP prostate cancer cells it does so without inducing cell cycle arrest or apoptosis (Hill & Kyprianou 2004).

Glycyrrhetinic acid (GA), one of the active components of liquorice, is known to have potent anti-inflammatory activity and has been used in the treatment of hepatic inflammation (Satomi et al 2005). Glycyrrhizic acid (GL) has also been shown to have a protective effect in hepatitis C patients, reducing their probability of developing hepatocellular carcinoma (Arase et al 1997). In in-vitro studies, GA and GL induced cell cycle G1 arrest and apoptosis in human hepatoma HepG2 cells.

Results seen in Figure 2 demonstrated that liquorice extract and GA had a significant effect on the rate of proliferation of androgen-dependent LNCaP cells, whereas they had no effect on the proliferation of PC3 and DU145 androgen-independent cells. The reduction in cellular proliferation seen when LNCaP cells were treated with liquorice or GA may have been due to the initiation of cell cycle arrest and induction of apoptosis, as was seen with hepatoma cell lines (Satomi et al 2005).

The results demonstrated that purified GA possessed more potent antiproliferative properties than liquorice extract. Liquorice extract contains a number of phytochemicals, including GA and flavonoids, which may work synergistically or possess antagonistic effects. The benefit of using purified GA is that the exact mechanism of action of the compound may be more easily determined as there are no other contributing factors; also the exact concentration of GA in each batch of liquorice extract is unknown and therefore it would be beneficial to use purified GA of known concentrations in subsequent studies.

A reduction in PSA levels was demonstrated in LNCaP cells after treatment with PC SPES, which was due to the inhibition of androgen-controlled activation of the PSA promoter/enhancer via inhibition of androgen receptor transcriptional activity (Ikezoe et al 2003). Other studies have suggested that PC SPES may cause an increase in PSA levels in LNCaP cells (Bonham et al 2002) and it has been hypothesized that PC SPES may possess biphasic activity dependent on dosage. Our results (Figure 3) have shown that treatment of LNCaP cells with either liquorice extract or GA significantly reduced the levels of secreted PSA, which may have been due to potential anti-androgenic properties of GA. Again the results demonstrated that purified GA had more potent effects than liquorice extract, possibly for the reasons mentioned previously. Reduction in PSA levels could prove beneficial since it is known to influence cell adhesion and ECM degradation (Killian et al 1993; Weber et al 1995; Lilja et al 2000; Romanov et al 2004).

Our results suggested that GA (and liquorice extract) may have a role to play in the modulation of androgen-dependent prostate cancer growth via inhibition of cell proliferation and down-regulation of PSA production. It is important to stress that these results were merely a starting block for our investigations and that extensive further investigation is required to determine which, if any, of these mechanisms may have been responsible for the biological activity. Future work will need to include assessment of GA antagonistic properties at the androgen receptor, measurement of androgen receptor levels

in GA-treated cells, determination of potential apoptotic pathways, e.g. bcl-2 and BAX, and use of flow cytometry to investigate potential cell cycle inhibition.

Whilst these are very early days in our investigations, the possibility exists that GA may have the potential to be used as an alternative or adjunct to current aggressive therapies for androgen-dependent prostate cancer management.

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