6-Carboxymethyl genistein: a novel selective oestrogen receptor modulator (SERM) with unique, differential effects on the vasculature, bone and uterus

D Somjen¹, Y Amir-Zaltsman, B Gayer, T Kulik, E Knoll¹,
N Stern¹, L J W Lu², L Toldo³ and F Kohen

Department of Biological Regulation, Weizmann Institute of Science, Rehovot, 76100 Israel

¹Institute of Endocrinology, Tel-Aviv Sourasky Medical Center and the Sackler Faculty of Medicine, Tel-Aviv University, Israel

²Department of Preventive Medicine and Community Health, The University of Texas Medical Branch, Galveston, Texas, USA

³Department of Bio and Chemoinformatics, Merck KGaA, Darmstadt, Germany

(Requests for offprints should be addressed to F Kohen; Email: fortune.kohen@weizmann.ac.il)

Abstract

The novel genistein (G) derivative, 6-carboxymethyl genistein (CG) was evaluated for its biological properties in comparison with G. Both compounds showed oestrogenic activity *in vitro* and *in vivo*. On the other hand G and CG differed in the following parameters: (i) only CG displayed mixed agonist-antagonist activity for oestrogen receptor (ER) α in transactivation assays and (ii) only CG was capable of attenuating oestrogen (E₂)-induced proliferation in vascular smooth muscle cells and of inhibiting oestrogen-induced creatine kinase (CK) specific activity in rat tissues. On the other hand only G enhanced the stimulatory effect on CK specific activity in the uterus. In comparison to the selective oestrogen receptor modulator (SERM) raloxifene (RAL), CG showed the same selectivity profile as RAL in blocking the CK response to E₂ in tissues derived from both immature and ovariectomized

female rats. Molecular modelling of CG bound to the ligand binding domain (LBD) of ER β predicts that the 6-carboxymethyl group of CG almost fits the binding cavity. On the other hand, molecular modelling of CG bound to the LBD of ER α suggests that the carboxyl group of CG may perturb the end of Helix 11, eliciting a severe backbone change for Leu 525, and consequently induces a conformational change which could position Helix 12 in an antagonist conformation. This model supports the experimental findings that CG can act as a mixed agonist-antagonist when E₂ is bound to its receptors. Collectively, our findings suggest that CG can be considered a novel SERM with unique effects on the vasculature, bone and uterus.

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Introduction

During the last ten years extensive research on genistein (4',5,7-trihydroxy-isoflavone) (Fig. 1) has documented two important observations, one from investigations on the factors responsible for the reduced incidence of breast, prostate and colon cancer in Southeast Asia and the other from the search for pharmacological agents that interfere with growth factor signalling pathways in normal and cancer cells (Barnes *et al.* 1996). Interest has also focused in recent years on the question whether specific components in the Asian diet may have cancer protective effects (Setchell 2001). Soybean and rice constitute the major diet in East-Asian countries, and soya contains significant amounts of the isoflavones daidzein and genistein (G) which may act as weak oestrogens or as anti-oestrogens. Of these two isoflavones, genistein has been studied a great deal due to its wide spectrum of biological activities,

including inhibition of tyrosine phosphorylation of proteins in the signal transduction pathway, inhibition of topoisomerase II, and induction of apoptosis and cell differentiation (Barnes 1997).

The factors determining whether the oestrogenicity or anti-oestrogenicity of isoflavones predominate are quite complex. These factors may be species and/or tissue specific, and may depend on the concentration of the isoflavone, the length of time used and the method of administration (Barnes *et al.* 2000). Moreover, G shows a greater binding affinity for oestrogen receptor (ER) β than for ER α (Kuiper *et al.* 1998). There is no plausible explanation so far for the distinct ligand binding preference of G for ER β . Additionally, in the three-dimensional structure of the ligand binding domain (LBD) of the ER β -G complex the transactivation Helix 12 does not adopt the agonist position, but, instead, lies in a similar orientation to that induced by the selective oestrogen

Figure 1 Structure of 6-carboxymethyl genistein (CG) and genistein (G).

receptor modulator (SERM) raloxifene (Pike *et al.* 1999). Such alignment of Helix 12 suggests that G is a partial agonist when bound to ER β (Pike *et al.* 2000, 2001). In view of the fact that G exhibits a wide variety of effects, several analogues of genistein have been synthesized and evaluated for their immunosuppressant and anti-tumour activity (Fiedor *et al.* 1998). In previous work we have described the synthesis of a novel derivative of G through position 6 of the molecule, 6-carboxymethyl genistein (CG) (Kohen *et al.* 1999) (Fig. 1), and evaluated its properties as a tyrosine kinase inhibitor in comparison with G. We found that CG did not affect tyrosine kinase activity (Amir-Zaltsman *et al.* 2000). We have continued our work in evaluating the oestrogenic and anti-oestrogenic properties of CG. In the present study the *in vitro* and *in vivo* oestrogenic and anti-oestrogenic properties of CG in comparison with genistein (G) are described. Our results indicate that CG shows mixed agonist-antagonist activity both *in vitro* and *in vivo*. In order to explain the observed anti-oestrogenic activity of CG, a computational investigation of the predicted orientation of CG within the LBD of ER α and ER β was undertaken. The docked model of CG within the LBD of ER β predicts that CG almost fits within the binding pocket. On the other hand, the docked model of CG within the LBD of ER α predicts that CG perturbs receptor structure by causing a severe backbone change for Leu 525 in Helix 11. This ligand-induced conformational change in the receptor structure may prevent correct alignment of Helix 12 for recruitment of coactivators (Egner *et al.* 2001) and cause the observed anti-oestrogenic effect of CG when oestrogen (E₂) is bound to ER α . Since CG opposes the effects of oestrogen in rat uterine tissue and shows agonistic effects in bone and vasculature, it can be considered a novel SERM.

Materials and Methods

Reagents

All reagents were of analytical grade. Chemicals, tamoxifen, steroids and biotin- ϵ -aminocaproyl- γ -butyryl-N-succinimide ester were purchased from Sigma (St

Louis, MO, USA). Isoflavones were purchased from Indofine Chemical Company (Somerville, NJ, USA). Baculovirus-expressed recombinant ER α and ER β preparations were from Panvera (Madison, WI, USA). Polyclonal anti-ER β antibody was purchased from Santa Cruz (Santa Cruz, CA, USA) and polyclonal anti-ER α antibody was a generous gift from Dr H Thole (Max-Planck Institute, Germany). [³H]17 β -Oestradiol (85 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Streptavidin-coated plates were from Wallac (Turku, Finland). Raloxifene was extracted into ethanol from commercially available tablets (Evista).

Synthesis of carboxy derivatives of isoflavones

6-Carboxymethyl genistein (CG) (see Fig. 1 for the structure) was prepared as described previously (Kohen *et al.* 1999).

Biotinylation of antibodies

Anti-ER antibodies were biotinylated with biotin- ϵ -aminocaproyl- γ -butyryl-N-succinimide ester as previously described (Strasburger & Kohen 1990).

Receptor-binding assays

Recombinant ER α or ER β proteins (12 pmol/ml) in 10 μ l binding buffer (10 mM Tris, pH 7.5, containing 10% glycerol, 2 mM dithiothreitol (DTT), and 1 mg/ml BSA) were incubated in streptavidin-coated microtitre plates for 30 min at room temperature, in the absence or presence of serial dilutions of oestrogen in 50 μ l binding buffer or test compounds. Subsequently, [³H]E₂ (3 nM) in 50 μ l binding buffer was added to each well, and the mixtures were incubated overnight at 4 °C. Biotinylated anti-ER antibody (α or β) (100 ng/well in 100 μ l binding buffer) was added to each well, and the reaction mixtures were incubated with shaking for 2.5 h at room temperature. The reaction mixtures were then decanted, and each well was washed once with binding buffer. Dilute sodium hydroxide (0.4 mM, 300 μ l) was added to each well. After shaking for 20 min, an aliquot (200 μ l) was removed from

each well and added to a vial containing scintillation fluid. The vials were then counted for radioactivity in a beta scintillation counter.

Transfection and luciferase assay

MCF-7 human breast cancer (National Cancer Institute, Bethesda, MD, USA) or human 293 embryonal kidney cells (American Type Tissue Collection (ATTC), Rockville, MD, USA) were plated in 24-well plates at a density of 50 000 cells/well in complete medium consisting of RPMI-1640 or DMEM containing phenol red supplemented with 10% fetal calf serum (FCS) (Biological Industries, Beth Haemek, Israel), 2% L-glutamine and antibiotics. Cells were incubated for 72 h at 37 °C in a humidified atmosphere of 8% CO₂-air. Afterwards, the medium of each well was aspirated, and each well received 0.5 ml phenol red-free RPMI-1640 or DMEM without FCS and antibiotics. The MCF-7 cells were then transfected following the Geneporter procedure (Gene Therapy Systems, San Diego, CA, USA) with three plasmids: (a) 3 xERE-TATA-Luc reporter plasmid (360 ng/well, from Dr Bart van der Burg, Utrecht, The Netherlands); (b) pEGFP-C1 (120 ng/well, from CLONETECHniques, Palo Alto, CA, USA) for visualization of transfection and (c) Renilla phRL (48 ng/well, from Promega, Madison, WI, USA) for normalizing the luciferase activity of the reporter plasmid in each well. The 293 cells were transfected with the same plasmids as the MCF-7 cells and a fourth plasmid containing the human ER α (pRST7-ER α from Dr D McDonnell, Durham, NC, USA; 8 ng/well) or ER β (pRST7ER β long form encoding 530 amino acids, from Dr D McDonnell; 8 ng/well) was introduced. The cells were first incubated with 25 μ l DNA and liposome solution for 3 h at 37 °C and subsequently, 0.5 ml phenol red-free RPMI-1640 or DMEM medium containing 20% charcoal-treated FCS and antibiotics were added to each well. After an overnight incubation at 37 °C, the transfected cells were washed twice with PBS and treated with various test chemicals in complete medium without phenol red and supplemented with 10% charcoal-treated fetal calf serum. After an overnight incubation, treated cells were rinsed with PBS. Firefly and Renilla luciferase activity were determined using the Dual Luciferase Reporter assay system (Stop and Glow from Promega) and a luminometer (M2010A) from Lumac (Landgraaf, Holland).

Cell cultures

Human umbilical artery vascular smooth muscle cells (VSMC) Human umbilical artery VSMC were prepared as previously described with minor modifications (Somjen *et al.* 1998). In brief, umbilical cords were collected shortly after delivery. The umbilical arteries were cleaned of blood and adventitia and then cut into 1–3 mm

slices. The segments were kept in culture in Medium 199 containing 20% FCS, glutamine and antibiotics. Cell migration was detectable within 5–7 days. Cells were fed twice a week and, upon confluence, trypsinized and transferred to 24-well dishes. Cells were used only at passages 1–3 when expression of smooth muscle actin was clearly demonstrable.

Endothelial cells (E304) E304 cells, an endothelial cell line derived from a human umbilical vein, were purchased from ATCC, and grown in Medium 199 containing 10% FCS, glutamine and antibiotics.

Assessment of DNA synthesis

Cells were grown until subconfluence and then treated with various hormones or agents as indicated. Twenty-two hours later, [³H]thymidine was added for two hours. Cells were then treated with 10% ice-cold trichloroacetic acid (TCA) for 5 min and washed twice with 5% TCA and then with cold ethanol. The TCA extracts were combined and dissolved in 0.3 ml 0.3 M NaOH, samples were taken and aliquots were taken for measurement of [³H]thymidine incorporation into DNA as previously described (Somjen *et al.* 1998).

Animals

Immature, 25-day-old, female Wistar rats were used at the age of 25 days. Ovariectomy was performed at the age of 25 days, and the various treatments were carried out 2 weeks after surgery. The colony was housed in air-conditioned quarters with light from 0500 to 1900 h and had access to Purina Laboratory Chow and water. All experiments were carried out according to the regulations of the committee of the experimental animals of the Weizmann Institute of Science.

Creatine kinase extraction and assay

In vitro Cells were treated for 24 h with the various hormones and agents as specified. Cells were then scraped off the culture dishes and homogenized by freezing and thawing three times in an extraction buffer as previously described (Somjen *et al.* 2000). Supernatant extracts were obtained by centrifugation of homogenates at 14 000 g for 5 min at 4 °C in an Eppendorf microcentrifuge. Creatine kinase (CK) was determined by a coupled spectrophotometric assay (Sigma) as described previously (Somjen *et al.* 2000). Protein was determined by Coomassie blue dye binding using bovine serum albumin as the standard.

In vivo Changes in CK specific activity in the epiphyseal cartilage, diaphyseal bone, uterus, pituitary, aorta, and left ventricle of the heart, induced by the short-term treatment with E₂, G and CG were studied in 25-day-old immature

or one-month-old ovariectomized female rats, two weeks after surgery (five animals per group using the doses indicated for each experiment). Rats injected intraperitoneally (i.p.) with 0.05% ethanol in PBS served as controls. The E₂ dose used for each rat was 5 µg, since this dose is optimal for bone tissue. The rats were killed by decapitation 24 h after i.p. injection. The various organs were removed and stored at -20 °C until processed for CK activity.

Statistical analysis

The significance of differences between the mean values obtained from experimental and controls rats was evaluated by the unpaired two-tailed Student's *t*-test and by analysis of variance (ANOVA).

Computer modeling of 6-carboxymethyl genistein bound to ERβ

A structural model of CG bound to the ligand binding domain (LBD) of human ERβ was built manually by altering the G structure as observed in 1 QKM (Pike *et al.* 1999) using SYBYL modules (Tripos Inc., St Louis, MO, USA). A carboxyl methyl group was added to position 6 of the isoflavone ring and oriented optimally in order to minimize unfavourable interactions. Energy minimization and docking were subsequently performed with the SYBYL modules, using default parameters. This model is named CG-ERβ-(alpha face) to indicate that the ring hydroxyl (position 5) and keto group (position 4) of CG (Fig. 1) point towards the alpha face (Pike *et al.* 2001) of the binding cavity.

Computer modeling of 6-carboxymethyl genistein bound to ERα

The model of CG bound to the LBD of human ERα was generated using SYBYL modules by superimposing the protein backbone of the CG-ERβ complex with that of 1 ERE (Brzozowski *et al.* 1997), followed by manual refinement of the CG's carboxyl orientation, energy minimization and docking. In this model the oxygen atom at position 4 of CG is directed towards the alpha face of the binding cavity and is referred to as CG-ER alpha face. Using this model CG was then rotated 180 degrees over a diagonal going through the phenolic hydroxyl group at position 4' and the hydroxyl group at position 7. The model was then energy minimized and docked as above. This is the model of CG-ERα through the beta face. Ligand protein contact (LPC) was used to generate final ligand-protein contact data (Sobolev *et al.* 1999). Connolly surfaces, computed by SYBYL, were chosen for visualising relevant LPC areas of the protein.

Table 1 Relative binding affinity (RBA) of genistein analogues for ERα and ERβ. The RBA for each competitor was calculated as the ratio of concentrations of oestrogen (E₂) and competitor required to reduce [³H]oestradiol by 50% (=ratio of IC₅₀ values). The RBA value for E₂ was arbitrarily set at 100. The IC₅₀ value for E₂ was 0.8 nM for ERα and 1 nM for ERβ

Compound	RBA (%)	
	ERα	ERβ
Oestradiol	100	100
Genistein	0.1	1
6-Carboxymethyl genistein	<0.01	0.2

Results

Oestrogen receptor binding studies

In binding assays, carried out as described in the Materials and Methods section, G and CG show a relative binding affinity (RBA) to ERβ of 1 and 0.2% respectively. On the other hand G shows an RBA of 0.1% to ERα while CG does not significantly inhibit the binding of [³H]oestradiol to ERα (Table 1).

Transcriptional activity of genistein analogues

The transactivation activity of G and CG was measured after incubation of transfected culture cells with G analogues (1 µM) in the absence or presence of E₂ (1 nM in MCF-7 and 293 cells transfected with ERα and 10 nM in 293 cells transfected with ERβ). G showed only strong agonistic activity in MCF-7 (Fig. 2A) and 293 cells transfected with both subtypes (Fig. 2B and C), and no inhibitory activity in the presence of E₂. On the other hand, the agonistic activity of CG was moderate in MCF-7 and 293 cells transfected with ERα and strong in 293 cells transfected with ERβ. Moreover, CG in the presence of E₂ in MCF-7 and 293 cells transfected with ERα displayed a mixed agonist-antagonist activity for ERα (Fig. 2A, B), and no antagonism could be detected in 293 cells transfected with ERβ (Fig. 2C). Under the same experimental conditions, tamoxifen (1 µM) showed antagonist activity only in MCF-7 and 293 cells transfected with ERα (Fig. 2A,B) and no antagonism in cells transfected with ERβ (Fig. 2C) could be detected.

Effects of genistein analogues on [³H]thymidine incorporation and CK specific activity in vitro

Human VSMC Both E₂ (0.3 nmol/l to 30 nmol/l) and G (0.3 nmol/l to 3000 nmol/l) had a biphasic effect on DNA synthesis. At a low concentration of E₂ (0.3 nmol/l), [³H]thymidine incorporation was stimulated, whereas higher concentrations of E₂ (>30 nmol/l) led to inhibition

Figure 3 Upper panel: the effect of E₂, G and CG alone on creatine kinase (CK) specific activity in vascular smooth muscle cells (VSMC). Results are means $\pm$ S.E.M. of 8–16 incubates from 2–4 experiments and are expressed as the ratio between the specific activity in hormone-treated and control cells. * $P < 0.05$, ** $P < 0.01$. Lower panel: the effect of E₂, G and CG alone on DNA synthesis as assessed by [³H]thymidine incorporation in VSMCs. Results are means $\pm$ S.E.M. of 8–16 incubates from 2–4 experiments and are expressed as the ratio between [³H]thymidine incorporation in hormone-treated and control cells. The dashed lines represent the control basal activity. * $P < 0.05$.

Figure 2 (left) Transcriptional activation of ER α and ER β with oestrogen (E₂), G (Gen) or CG. MCF-7 human breast cancer cells were transfected with three plasmids consisting of 3xERE-TATA-Luc reporter, green protein (pEGFP-C1) and Renilla luciferase. 293 kidney embryonic cells were transfected with the same three plasmids as the MCF-7 cells and in addition a fourth plasmid containing the cDNA for human ER α or ER β was introduced. The cells were then treated with E₂ (10^{-9} M (MCF-7 and 293 cells transfected with ER α) or 10^{-8} M (293 cells transfected with ER β)), G (1 μ M), CG (1 μ M), tamoxifen (Tam, 1 μ M), or combinations of E₂+G, E₂+CG, E₂+Tam. Firefly luciferase activity was performed 24 h later. Luciferase activity was standardized to the internal co-transfected Renilla activity and expressed as the ratio of induced activity to the activity in the absence of ligand. Each data point represents the mean $\pm$ S.D. of triplicates of 3 different experiments.

Table 2 Concentration dependent interactions of genistein analogues with E₂ or raloxifene (RAL) in terms of DNA synthesis or CK specific activity in human vascular smooth muscle cells. Results are means ± S.E.M. of 8–16 incubates from 2–4 experiments and are expressed as the ratio between [³H]thymidine incorporation in hormone-treated and control cells or as the ratio between the specific activity of CK in hormone-treated and control cells

Compound	DNA synthesis	CK specific activity
Low dose		
E ₂ (0.3 nM)	1.76 ± 0.17**	1.52 ± 0.08*
Ral (30 nM)	1.77 ± 0.11**	1.57 ± 0.17*
Ral (30 nM)+E ₂ (0.3 nM)	1.73 ± 0.17**	0.90 ± 0.11
G (30 nM)	1.78 ± 0.13**	3.02 ± 0.16**
G (30 nM)+Ral (30 nM)	1.93 ± 0.15**	1.28 ± 0.15
G (30 nM)+E ₂ (0.3 nM)	2.82 ± 0.18***	3.59 ± 0.11***
CG (30 nM)	1.90 ± 0.1**	1.85 ± 0.2*
CG (30 nM)+Ral (30 nM)	1.62 ± 0.19*	1.08 ± 0.12
CG (30 nM)+E ₂ (0.3 nM)	1.02 ± 0.18	1.12 ± 0.18
High dose		
E ₂ (30 nM)	0.65 ± 0.16*	2.49 ± 0.15**
Ral (3 μM)	0.47 ± 0.11**	1.69 ± 0.13**
Ral (3 μM)+E ₂ (30 nM)	0.56 ± 0.16*	1.46 ± 0.28*
G (3 μM)	0.59 ± 0.11**	3.43 ± 0.22***
G (3 μM)+Ral (3 μM)	0.53 ± 0.12*	0.91 ± 0.12
G (3 μM)+E ₂ (30 nM)	0.48 ± 0.13**	5.23 ± 0.31***
CG (3 μM)	1.76 ± 0.11**	2.54 ± 0.35**
CG (3 μM)+Ral (3 μM)	0.86 ± 0.31	1.28 ± 0.18
CG (3 μM)+E ₂ (30 nM)	0.96 ± 0.12	1.18 ± 0.25

Low dose: cells were treated with either G (30 nM) or CG (30nM) alone or in the presence of RAL (30 nM) or E₂ (0.3 nM) and assayed for [³H]thymidine incorporation and CK specific activity. High dose: cells were treated with either G (3 μM) or CG (3 μM) alone or in the presence of RAL (3 μM) or E₂ (30 nM) and assayed for [³H]thymidine incorporation and CK specific activity. *P<0.05, **P<0.01, ***P<0.005.

of DNA synthesis (Fig. 3, lower panel). Similar dose-related effects were seen with G, i.e. stimulation with a low concentration (30 nmol/l) and inhibition with high concentrations (>3 μmol/l) (Fig. 3, lower panel). On the other hand, CG had a monophasic stimulatory effect on DNA synthesis and exerted no inhibitory effect at high concentrations (Fig. 3, lower panel). Additionally, cells were more sensitive to CG than to G (two orders of magnitude). Interestingly, both compounds at 30 nmol/l increased significantly the CK specific activity in these cells, but this was maximal at 300 nmol/l (Fig. 3 upper panel).

In order to study further the anti-oestrogenic activities of CG in VSMCs the dose-dependent interactions of G or CG with E₂ or the SERM raloxifene (RAL) were investigated. The combination of G at low dose (30 nM) with E₂ (0.3 nM) augmented the response to E₂ by VSMC in terms of DNA synthesis and CK specific activity (Table

Figure 4 The effect of E₂ (30 nM), G (3 μM), CG (300 nM) or raloxifene (RAL 3 μM) alone or in the presence of either raloxifene (RAL 3 μM) or E₂ (30 nM) on DNA synthesis (upper panel) and on CK (lower panel) in E304 cells. Results are means ± S.E.M. of 8–16 incubates from 2–4 experiments and are expressed as the ratio between [³H]thymidine incorporation (DNA) or the specific activity of CK in hormone-treated and control cells. *P<0.05, **P<0.01. The control basal activity is shown as a dashed line.

2). The combination of CG (30 nM) with E₂ (0.3 nM) caused an inhibition of both cell proliferation and stimulation of CK specific activity (Table 2). On the other hand, combination of G at high dose (3 μM) with E₂ (30 nM) caused slightly increased suppression of cell proliferation and an increased stimulation in CK specific activity. The combination of CG at low or high dose with E₂ had an inhibitory effect on CK specific activity (Table 2).

In the next set of experiments the inhibitory effects of the SERM RAL were studied. RAL inhibited CK induction by either high or low doses of E₂, G and CG (Table 2). On the other hand, DNA synthesis induced by E₂ or G at high or low dose was unaffected by RAL (Table 2). Only CG-induced DNA synthesis at high doses was blocked by RAL (Table 2).

Figure 5 The effect of E₂ (5 µg), G (0.5 mg), CG (50 µg) and RAL (0.5 mg) alone or in the presence of RAL (0.5 mg) or E₂ (5 µg) on CK in epiphyseal cartilage (Ep) and diaphyseal bone (Di) in immature (upper panel) or ovariectomized (lower panel) female rats. Results are means ± S.E.M. of 5–15 assays from 2–3 experiments and are expressed as the ratio between the specific activity of CK in hormone-treated and control animals. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Endothelial cells (E304) E₂ (0.3 nmol/l), G (30 nmol/l) and CG (30 nmol/l) increased both DNA synthesis (Fig. 4, upper panel) and CK specific activity (Fig. 4, lower panel) in these cells. Combination of E₂ with G further augmented the response to E₂ with respect to DNA synthesis and CK specific activity. On the other hand CG in the presence of E₂ inhibited both cell proliferation and CK activity. Raloxifene (RAL) inhibited both DNA and CK induced by E₂, G or CG (Fig. 4).

Stimulation of the CK specific activity by genistein analogues in vivo

Immature and ovariectomized female rats were injected with E₂ (5 µg/rat), G (0.5 mg/rat), CG (100 µg/rat) or RAL (0.5 mg/rat) or with the combination of E₂+G, E₂+CG, E₂+RAL, G+RAL or CG+RAL. E₂ and G stimulated the CK specific activity in all the rat tissues (epiphyseal cartilage and diaphyseal bone (Fig. 5, upper and lower panel), pituitary and uterus (Fig. 6, upper and

Figure 6 The effect of E₂ (5 µg), G (0.5 mg), CG (50 µg) and RAL (0.5 mg) alone or in the presence of RAL (0.5 mg) or E₂ (5 µg) on CK specific activity in pituitary (Pi) and uterus (Ut) in immature (upper panel) or ovariectomized (lower panel) female rats. Results are means ± S.E.M. of 5–15 assays from 2–3 experiments and are expressed as the ratio between the specific activity of CK in hormone-treated and control animals. **P*<0.05, ***P*<0.01.

lower panel), left ventricle of the heart and aorta (Fig. 7, upper and lower panel)) that were tested, while CG increased the CK specific activity in all the rat tissues with the exception of the uterus (Fig. 6). On the other hand, RAL was stimulatory in the diaphysis, epiphysis, pituitary, aorta and left ventricle but not in the uterus. The stimulatory response of E₂ to CK specific activity was inhibited in all the tissues when rats were treated with a combination of E₂ plus CG or E₂ plus RAL (Figs 5–7), suggesting that CG acts like a SERM in these tissues. Moreover, the combination of E₂ plus G augmented the

CK response to E₂ in the aorta and pituitary and not in the remaining rat tissues. When rats were treated with G or CG plus RAL, the CK response to G or CG was blocked in all the tissues tested (Figs 5–7).

Computer model of the 6-carboxymethyl genistein bound to ERβ

The docked model of the LBD-ERβ-CG complex is shown in Fig. 8. In this model the phenolic group at position 5 and the keto group at position 4 of CG point

towards the alpha face of the binding cavity. The 6-carboxymethyl group in CG is bumping against the floor of the cavity made by Ile 373 and Ile 376. CG almost fits the binding cavity and can adapt slightly in the binding pocket since the small size of Ile residues do not decrease the volume and polarity of the binding pocket.

Computer model of 6-carboxymethyl genistein bound to ER α

The docked model of the LBD-ER α -CG complex and the receptor bound conformation observed for 4-OH tamoxifen pointing towards the beta face of the binding cavity are illustrated in Fig. 9. The docked geometries for CG and 4-OH tamoxifen suggest that the same amino acid residues (Arg 394 and Glu 353) in the LBD of ER α are involved in anchoring CG through the phenolic group at position 4' and tamoxifen through the 4-OH group (Pike *et al.* 2000). On the other hand, the two structures differ with respect to the orientation of side chains. In 4-OH tamoxifen the side chain exits through Helix 3 and 11 whereas in CG the 6-carboxymethyl group perturbs the end of Helix 11. This perturbation may lead to a severe backbone change for Leu 525 which may result in an antagonist conformation of Helix 12 (Fig. 9). The model shown in Fig. 9 predicts that CG fits very tightly within the binding pocket of ER α .

Discussion

In this paper we characterize a novel derivative of genistein (G), 6-carboxymethyl genistein (CG), in terms of binding to ER α and ER β and describe the transcriptional and agonistic and antagonistic effects of CG *in vitro* and *in vivo* in comparison to G. In addition, computer modeling of the LBD-ER α -CG complex and the LBD-ER β -CG complex is also used in order to correlate the structural models with the experimental findings.

The introduction of a carboxymethyl group to position 6 of G causes the derivative CG to bind selectively to ER β , but minimally to ER α . This difference may be due to the amino acid in ER α and ER β interacting with the ligand. The contact amino acids are Ile at position 373 in ER β and most probably Met 421 in ER α . It is known that Met 421 causes a decrease in the volume and polarity of the binding cavity (Pike *et al.* 1999) where the carboxymethyl group fits. It is suggested here that the minimal binding of CG to ER α (see Table 1) may be due to the presence of Met 421 in the LBD of ER α .

Both compounds G and CG were capable of inducing transcription via an oestrogen receptor element in MCF-7 cells that possess endogenous ER α , and in 293 cells transfected with ER α or ER β . However, only CG and not G was capable of displaying mixed agonist antagonist activity for ER α in MCF-7 and in 293 cells (Fig. 2A and B). No antagonism of CG could be detected in cells

transfected with ER β (Fig. 2). Under the same experimental conditions tamoxifen showed antagonistic effects only for ER α and not for ER β (Fig. 2).

When the ability of CG and G to stimulate DNA synthesis was assessed in VSMCs, the cells were more sensitive to CG than G by two orders of magnitude (Fig. 3, lower panel). Additionally CG lacked the inhibitory action on cell growth exerted by high concentrations of G. Combination of a low dose of G (30 nM) with a low dose of E $_2$ had no effect on the response of E $_2$ with respect to DNA synthesis (Table 2). Moreover, CG at high dose blocked the inhibitory effect on proliferation seen at high concentrations of E $_2$ (Table 2). Interestingly both G and CG at 30 nmol/l increased the CK response in VSMCs although the highest stimulation was seen at 300 nmol/l (Fig. 3, upper panel), and only CG at low and high doses attenuated the CK response in these cells in the presence of E $_2$ (Table 2). The SERM RAL inhibited the CK response to E $_2$, G and CG at both low and high doses (Table 2). RAL inhibited DNA synthesis induced by CG at high dose and did not affect the stimulation or suppression of the DNA synthesis induced by E $_2$ or CG (Table 2).

When the effects of G and CG were examined in E304 cells both compounds stimulated DNA synthesis and CK response in these cells (Fig. 4). However, the combination of E $_2$ with G caused an augmentation of the response in both DNA synthesis and CK induction whereas the combination of E $_2$ with CG caused an attenuation of proliferation and CK activity (Fig. 4). In these cells RAL inhibited both DNA synthesis and CK induced by either E $_2$ or G or CG (Fig. 4).

In experiments using immature or ovariectomized female rats both G and E $_2$ stimulated CK specific activity in all the tissues tested (epiphyseal cartilage, diaphyseal bone, pituitary, uterus, left ventricle of the heart and aorta) (Figs 5–7). Moreover, similar to RAL CG stimulated CK specific activities in all these tissues with the exception of the uterus (Figs 5–7). When rats were treated with a combination of E $_2$ (0.5 μ g/rat) plus CG (100 μ g/rat) or E $_2$ (0.5 μ g/rat) plus RAL (0.5 mg/rat), the stimulatory response to E $_2$ was attenuated in all the rat tissues tested (Figs 5–7), thus indicating that CG acts like a SERM in these tissues. When the rats were treated with a combination of E $_2$ plus G, an increase in CK was observed only in the aorta and pituitary. Moreover, treatment of rats with RAL plus G or RAL plus CG caused an inhibition of CK response in all tissues tested (Figs 5–7). Compared with RAL, CG is as effective at 5 to 10 times lower concentrations in blocking the CK response to E $_2$ in rat tissues *in vivo*.

Pending crystallographic evidence of CG complexed to ER α and to ER β limited computer modelling was performed in order to suggest an hypothesis on the anti-oestrogenic properties of CG. The modelling was performed in a very conservative way, choosing not to model protein fluctuations, but merely the orientation of

Figure 7 The effect of E₂ (5 µg), G (0.5 mg), CG (50 µg) and RAL (0.5 mg) alone or in the presence of RAL (0.5 mg) or E₂ (5 µg) on CK specific activity in the left ventricle of the heart (Lv) and the aorta (Ao) in immature (upper panel) or ovariectomized (lower panel) female rats. Results are means ± S.E.M. of 5–15 assays from 2–3 experiments and are expressed as the ratio between the specific activity of CK in hormone-treated and control animals. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

the ligand in the binding cavity. Since the 3D structure of the LBD–ERβ–G complex has been determined (Pike *et al.* 1999), a carboxymethyl group at the 6 position of genistein was added manually. Energy minimization and docking were subsequently performed using the SYBYL modules. Figure 8 illustrates the LBD–ERβ–CG complex with the phenolic hydroxyl group at position 5 and the keto group at position 4 pointing towards the alpha face of the binding pocket. It is evident from the model that CG almost fits in the binding pocket. This may be

due to the presence of Ile 373 which enables ERβ to accommodate more polar substituents at the distal end of the cavity.

So far, all the side chains of the anti-oestrogens (e.g. 4-OH tamoxifen, raloxifene) have been positioned through the 11β channel (Pike *et al.* 1999). The carboxymethyl group in CG is not long enough to stick into the 11β channel. Instead, in the simple model of LBD–ERα–CG through the beta face (Fig. 9) the carboxymethyl group at the 6-position of CG interacts with Leu

Figure 8 Docked 'model structure' obtained for CG bound to the ligand binding domain of ER β through the alpha face. This model was rendered with SYBYL 6-7. Oxygen atoms are depicted red, the oestrogen receptor green and interacting amino acids yellow.

525, a key residue of Helix 11. It is known (Gangloff *et al.* 2001) that Leu 525 adjusts its position to compensate the ligand's different binding modes. In that same study (Gangloff *et al.* 2001) it has been shown that disruption of Helix 11 terminus results in lengthening of loop 11–12 and allows Helix 12 to reach the coactivator binding groove. Therefore, it can be hypothesized from the model that CG binds in ER α in the beta face and in this way CG disturbs the terminus of Helix 11, eliciting a conformational change in the orientation of Helix 12. This hypothesis is consistent with the generally accepted mechanism of 'antagonism' in ER, as reported by Gangloff *et al.* (2001), that steric hindrance inhibits the natural agonist conformation and favours an alternative position for Helix 12.

Given the correlation between the model structure of CG within the LBD of ER α and the observed anti-oestrogenic activities, the docked model of LBD–ER α –CG complex shown in Fig. 9 suggests a new mechanism for eliciting an anti-oestrogenic response.

In summary, introduction of a carboxymethyl group at the 6-position of genistein imparts anti-oestrogenic properties to CG, and causes CG to act as a mixed antagonist both *in vitro* and *in vivo*. Since CG shows no oestrogenic activity in the uterus in terms of stimulation of CK activity, blocks the response of E₂ to CK in the uterus and shows oestrogenic activity at low doses in the bone and vasculature, CG can be considered a novel SERM.

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Figure 9 Docked 'model structure' obtained for CG bound to the ligand binding domain of ER α through the beta face. Note the 6-carboxymethyl of CG interacts with Leu 525 causing a severe backbone change at the end of Helix 11. For comparison purposes tamoxifen has been superimposed. The structures are coloured yellow for CG and blue for tamoxifen. Oxygen atoms are depicted red, the oestrogen receptor green and interacting amino acids yellow. This model was rendered with SYBYL 6.7.

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