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Genistein and other soya isoflavones are potent ligands for transthyretin in serum and cerebrospinal fluid

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Consumption of soya-based nutrients is increasing in modern society because of their potentially protective effects against chronic diseases. Soya products are also heavily advertised as alternative drugs for relief from symptoms of the menopause and for hormone replacement therapy. However, because of their oestrogenic activity, negative effects of isoflavones have been postulated. Therefore, we analysed influences of soya isoflavones, major soya constituents with endocrine activity, on thyroxine (T_4) binding to its distribution proteins. Serum binding of ^{125}I-labelled L-T_4 was analysed in the absence or presence of increasing concentrations of soya isoflavones using non-denaturing PAGE for analysis. Complete displacement of [^{125}I]T_4 binding to transthyretin (TTR) was observed in human serum incubated with genistein at concentrations $>10\,\mu$M; interference started at $>0{\cdot}1\,\mu$M. Glycitein showed decreased and daidzein the lowest displacement potency. [^{125}I]T_4 was displaced to albumin in rat and to T_4-binding globulin in human serum. Soya isoflavones also obstruct [^{125}I]T_4 binding to TTR in human cerebrospinal fluid (CSF). The inhibitory effect was confirmed in direct binding assays using purified TTR with 50 % inhibitory concentration values of $0{\cdot}07\,\mu$M for genistein, $0{\cdot}2\,\mu$M for glycitein and $1{\cdot}8\,\mu$M for daidzein. The present study underlined a potent competition of soya isoflavones for T_4 binding to TTR in serum and CSF. Isoflavones might alter free thyroid hormone concentrations resulting in altered tissue availability and metabolism. As a consequence of this interference, one could expect a disturbance in the feedback regulation of hormonal networks, including the pituitary–thyroid–periphery axis during development and in adult organisms.

Thyroxine: Transthyretin: Genistein: Isoflavones: Cerebrospinal fluid

Several experimental and clinical studies have shown that consumption of soya and soya food products have beneficial effects on human health. Particularly, studies on soya isoflavones revealed their possible protective role against different forms of cancer, osteoporosis, CVD and renal disease (Tham *et al.* 1998; Messina, 1999; Anderson *et al.* 1999; Lissin & Cooke, 2000; Jin & MacDonald, 2002; Cross *et al.* 2004; Duncan *et al.* 2005). Their structural similarities to 17β-oestradiol, and ability to preferentially bind to oestrogen receptor β (50 % inhibitory concentration (IC_{50}) values of 8·4 nM for genistein and 100 nM for daidzein) and sex hormone-binding globulin (Kuiper *et al.* 1998; Dixon & Ferreira, 2002; Doerge & Sheehan, 2002), identified these soya isoflavones as acting as potential selective oestrogen receptor modulators, this being the reason for their wide but controversial use in relieving postmenopausal symptoms in women.

Despite the numerous beneficial effects of soya isoflavones, epidemiological and experimental data also exist showing an adverse effect on human health. Soya isoflavones exhibit oestrogen activity but, administered during development, can cause adverse oestrogen effects in experimental animals (Dixon & Ferreira, 2002). The main isoflavone genistein also inhibits tyrosine kinase (IC_{50} about $150\,\mu$M) and other protein kinases by acting as a competitive inhibitor of ATP binding at higher doses than needed for oestrogen receptor binding (Akiyama *et al.* 1987). Recently it was found that mice neonatally exposed to genistein develop uterine cancer later in their life, reminiscent of certain effects of oestrogen analogues, such as diethylstilbestrol (Newbold *et al.* 2001). The negative effects of soya on the pituitary–thyroid axis are also well described in human subjects and animals. The studies on rats revealed associations between goitrogenesis and soya consumption, and the protective effect of adequate dietary iodide intake (Block *et al.* 1961; Nordsiek, 1962; Konijn *et al.* 1972; Kay *et al.* 1988). Hypothyroidism and goitre were also observed in infants consuming soya formula (van Wyk *et al.* 1959; Hydovitz, 1960; Shepard *et al.* 1960; Ripp, 1961; Pinchera *et al.* 1965; Labib *et al.* 1989; Chorazy *et al.* 1995; Jabbar *et al.* 1997); however, goitre was reversed after switching to cows' milk or iodine supplementation.

There are at least three different levels at which soya isoflavones can interact with the thyroid hormone system: at the thyroid gland; in metabolism (with feedback mechanisms); with thyroid hormone transport proteins (Köhrle, 2000). Genistein and daidzein were identified as potent inhibitors of thyroid peroxidase, a key enzyme in thyroid hormone synthesis, *in vitro* and *in vivo* (Divi *et al.* 1997; Chang & Doerge, 2000; Doerge & Sheehan, 2002). They block both

Abbreviations: CSF, cerebrospinal fluid; IC_{50}, 50 % inhibitory concentration; T_4, thyroxine; TBG, thyroxine-binding globulin; TTR, transthyretin.

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iodination of tyrosine residues on thyroglobulin and the coupling of two iodinated tyrosine molecules to yield iodothyronines. Daidzein and genistein also affect the metabolism of thyroid hormones and iodide re-utilisation in the human thyroid by inhibition of sulfotransferase enzymes (Ebmeier & Anderson, 2004).

In the healthy adult rat, thyroid hormones are transported to target tissues primarily bound to transthyretin (TTR), the major serum distributor protein for thyroid hormone in rodents (Young *et al.* 1982). This is in contrast to human serum, where thyroxine-binding globulin (TBG) binds thyroxine (T_4) with highest affinity. Flavonoids are strongly and preferentially bound to TTR in most species, including man, but show no or only minor competition with thyroid hormone for binding to TBG, or to serum albumin (Köhrle *et al.* 1989). In the present *in vitro* study, we report the influence of soya isoflavones (Fig. 1) on thyroid hormone binding and distribution, in particular on the binding of T_4 to its distributor proteins in human and rat serum as well as in human cerebrospinal fluid (CSF).

Materials and methods

Chemicals and materials

^{125}I-labelled L-T_4 (specific activity 4·99–6·10 MBq/μg) was purchased from Perkin Elmer (Billerica, MA, USA). 3,3′,5,5′-Tetraiodo-L-T_4 was kindly provided from Henning Berlin (Germany). Human purified TTR and TBG were prepared by Vivian Cody (Hauptman-Woodward Medical Research Institute, Buffalo, NY, USA). The soya isoflavone genistein was purchased from Sigma-Aldrich (Germany) and isoflavones daidzein and glycitein were kindly provided by Sabine Kulling (Karlsruhe, Germany). The Wistar rat serum pool was kindly provided by Franziska Götz (Institute of Experimental Endocrinology, Charité, Berlin, Germany) and the human serum pool and CSF pool from Lutz Schomburg and Ulrich Schweizer (Institute of Experimental Endocrinology, Charité, Berlin, Germany), respectively.

Analysis of thyroxine binding to serum proteins

Binding of ^{125}I-labelled T_4 to serum and CSF proteins was assessed by non-denaturing PAGE, as previously described (Young *et al.* 1982). The sera and CSF samples (10 μl) were incubated in 1·5 ml Eppendorf tubes for 30 min at room temperature with 10 μl [^{125}I]T_4 (about 740 Bq) diluted in 0·02 M-phosphate buffer (pH 9) in the absence or presence of increasing concentrations of soya isoflavones (0·1–100 μmol/l). Samples (60 μl) of the incubated mixture were loaded on non-denaturing PAGE gels and run for 14 h at 50 V in a tri(hydroxymethyl)-aminomethane-glycine native running buffer (pH 8·4). The temperature was maintained at 6°C by the Bio-Rad Protean II xi cooling electrophoresis chamber (Bio-Rad Laboratories, Hercules, CA, USA). Gels were sealed in a plastic transparent bag and exposed to phosphoimager plates overnight, before scanning. The distribution of radiolabelled T_4 to individual binding proteins was analysed and quantified by a Cyclone storage phosphor screen (Packard Instrument Company Inc., Meriden, CT, USA).

In vitro *thyroxine–transthyretin competition-binding studies*

The analysis of the capacity of soya isoflavones to compete with T_4 binding to purified human TTR was performed as described previously (Somack *et al.* 1982), with slight modifications (Auf'mkolk *et al.* 1986). The assay mixture was a 0·1 M-tri(hydroxymethyl)-aminomethane-HCl buffer (pH 8·0) containing 0·1 M-NaCl and 1 mM-EDTA, purified human TTR (2·5 μg/μl = 23 nmol/l), ^{125}I-labelled L-T_4 (610 Bq/tube, about 50 000 cpm) and competitors (soya isoflavones) with increasing concentrations (0·001–10 μmol/l), in a total volume of 100 μl. Control incubations contained 1 % dimethylsulfoxide, which was the solvent, instead of the competitor. The incubation mixtures were allowed to reach binding equilibrium at room temperature for 30 min, and incubation was stopped by adding 0·5 ml ice-cold dextran-coated charcoal. TTR-bound and free [^{125}I]T_4 were separated after 10 min of incubation at 4°C by 10 min centrifugation at 3000 ***g***. The decanted supernatant fraction was counted in an LKB Wallac 1277 γ counter (Wallac, Milton Keynes, Bucks, UK). Unspecific binding, determined by adding L-T_4 (10 μmol/l), was subtracted to obtain specific binding data. All analyses were performed with data from at least three different experiments performed in duplicate. Calculation of binding parameters was performed with GraphPad Prism version 4 for Windows (GraphPad Software Inc., San Diego, CA, USA).

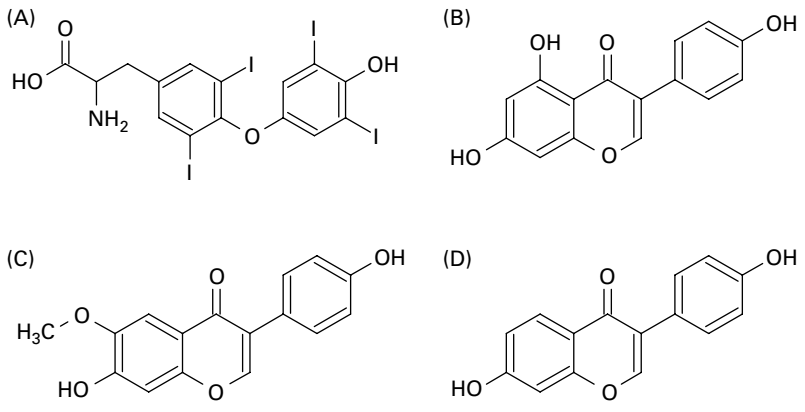

Fig. 1. Structural formulas of thyroxine (A) and soya isoflavones used in the study: genistein (B); glycitein (C); daidzein (D).

Results

Increasing concentrations of soya isoflavones added to human and rat serum and human CSF progressively inhibited the binding of ^{125}I-labelled L-T_4 to TTR. Native PAGE images presented in Fig. 2 show a representative example of the most potent competitor, genistein. Complete inhibition of [^{125}I]T_4 binding to TTR was achieved at a concentration of >10 μmol genistein/l in human serum with interference starting at $>0{\cdot}1$ μM concentrations (Fig. 2 (A)). The labelled hormone was displaced from TTR to TBG and albumin. The displacement potency decreased with the decrease in concentration of the competitor.

Fig. 2 (B) shows the effect of genistein on [^{125}I]T_4 binding to TTR in human CSF, which contains no TBG or albumin. Complete and marked displacement was observed at concentrations of 100 and 10 μmol/l respectively, leading to an increased amount of free [^{125}I]T_4 (lanes 5 and 6). Genistein added to pooled rat serum markedly inhibited the [^{125}I]T_4 binding to TTR at the concentrations of 100 and 10 μmol/l (Fig. 2 (C)). The labelled hormone was displaced from TTR to albumin. The other soya isoflavones, glycitein and daidzein, influence the [^{125}I]T_4 binding to serum and CSF proteins in the same manner, but with lower potency (data not shown). Purified TTR presented in Fig. 2 (A) (lane 3) was slightly shifted compared with native TTR, either because it was partially 'denatured' or devoid of retinol-binding protein after purification.

The binding of [^{125}I]T_4 to TTR in the presence of saturating concentrations of soya isoflavones is presented in Fig. 3. The sigmoidal dose–response curves describe the relationships between the isoflavones' concentration and [^{125}I]T_4 bound to human purified TTR. Genistein was the strongest competitor, showing practically the same displacement as the unlabelled L-T_4 used as a control in each experiment ($IC_{50} = 0{\cdot}07$ and $0{\cdot}08$ μM, respectively). Glycitein inhibited [^{125}I]T_4 binding to TTR with about four times less potency ($IC_{50} = 0{\cdot}2$ μM) and daidzein was the weakest competitor for binding to TTR ($IC_{50} = 1{\cdot}8$ μM). Scatchard analysis of the representative binding data yielded the following dissociation constants: T_4 (65 (SD 19) nM); genistein (59 (SD 13) nM); glycitein (71 (SD 22) nM); daidzein (131 (SD 109) nM).

Discussion

The results presented in the present *in vitro* study clearly demonstrate the inhibitory effect of soya isoflavones on the binding of T_4 to the serum and CSF thyroid hormone transport

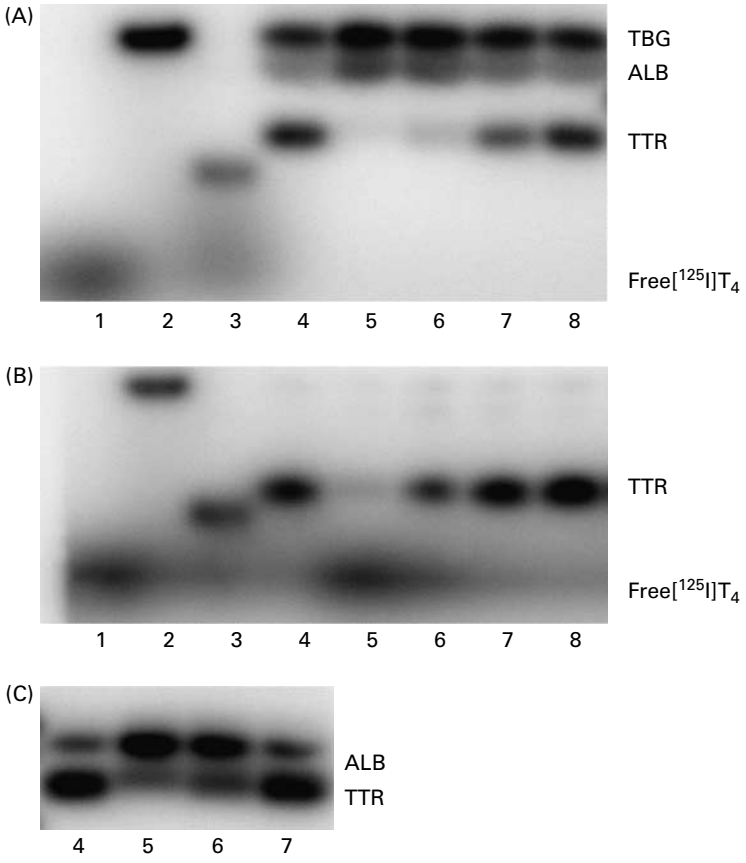

Fig. 2. Representative autoradiographs of [^{125}I]thyroxine ([^{125}I]T_4) bound to serum and cerebrospinal fluid (CSF) thyroid hormone-binding proteins. Effect of the isoflavone genistein added *in vitro* on binding of [^{125}I]T_4 to binding proteins in (A) human serum, (B) human CSF and (C) rat serum. In (A) the lanes are: lane 1, free [^{125}I]T_4; lane 2, purified human thyroxine-binding globulin (TBG); lane 3, purified human transthyretin (TTR); lane 4, control human serum; lane 5, human serum +100 μM-genistein; lane 6, human serum +10 μM-genistein; lane 7, human serum +1 μM-genistein; lane 8, human serum +0·1 μM-genistein. In (B) the lanes are: lane 1, free [^{125}I]T_4; lane 2, purified human TBG; lane 3, purified human TTR; lane 4, control human CSF; lane 5, CSF +100 μM-genistein; lane 6, CSF +10 μM-genistein; lane 7, CSF +1 μM-genistein; lane 8, CSF +0·1 μM-genistein. In (C) the lanes are: lane 4, control rat serum; lane 5, rat serum +100 μM-genistein; lane 6, rat serum +10 μM-genistein; lane 7, rat serum +1 μM-genistein. ALB, albumin.

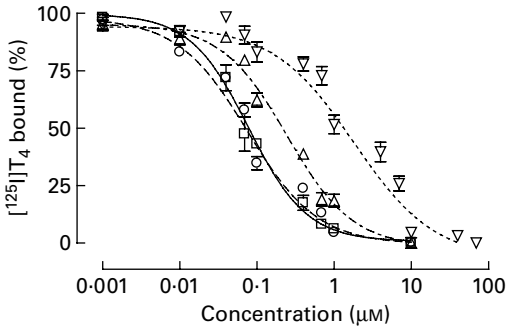

Fig. 3. Dose–response displacement of [^{125}I]thyroxine ([^{125}I]T_4; □) from transthyretin (TTR) by soya isoflavones genistein (○), glycitein (Δ) and daidzein (▽). Data points are mean values of at least three measurements in duplicate, with standard deviations represented by vertical bars. [^{125}I]T_4–TTR binding data were normalised for each experiment to span the range from 0 to 100 %. Values for 50 % inhibitory concentration (μM) were: T_4, 0·08; genistein, 0·07; glycitein, 0·2; daidzein 1·8.

protein, TTR. As no competition was observed for the binding of [^{125}I]T_4 to albumin and TBG (data not shown), the addition of soya isoflavones to rat and human serum resulted in the displacement of [^{125}I]T_4 from TTR to these transport proteins. Displacement also occurred in human CSF, but because of the absence of other specific binding proteins for T_4, the free [^{125}I]T_4 fraction was evidently increased. Genistein was the strongest competitor, showing binding affinity comparable with that of unlabelled T_4. Glycitein and daidzein exhibited lower competition potency. Selective binding of genistein and daidzein to TTR in plasma determined by an antibody capture–HPLC method, as recently published by Green *et al.* (2005), is in agreement with the present data.

The results presented in the present study are highly relevant both biologically and medically because the obtained IC_{50} concentrations are in the range of published soya isoflavone plasma concentrations. Doerge & Sheehan (2002) reported that adults eating typical Asian diets have blood concentrations of 0·1–1·2 μmol total soya isoflavones/l. Three adult volunteers had concentrations of 0·5–0·9 μmol/l after eating soya nutritional supplements and even 2–7 μmol/l concentrations of soya isoflavones were measured in the serum of seven infants eating soya infant formula. Manach *et al.* (2005) reviewed ninety-seven polyphenol bioavailability studies and showed similar results; for example, concentrations of 1·74 and 1·33 μmol genistein/l were measured in plasma 6 h after intake of soya milk and tofu, respectively. As these values are far above IC_{50} values obtained *in vitro* for genistein (0·07 μmol/l) and even glycitein (0·2 μmol/l), displacement of T_4 from TTR could be equally possible *in vivo*. Altered binding of T_4 to TTR, associated with altered free thyroid hormone levels, might be possible and might exert physiological effects. Watanabe *et al.* (2000) reported altered T_4 and triiodothyronine values in young premenopausal women, after administration of physiological doses of isoflavones. Similar doses of soya isoflavones caused only modest hormonal effects in postmenopausal women (Duncan *et al.* 1999). Selective binding of (iso)flavonoids to TTR might also indicate a role of TTR in distribution and targeting of soya isoflavones to steroid-regulated tissues, an effect independent of T_4 competition. As reported by Cassidy *et al.* (1994) and Watanabe *et al.* (2000), physiological doses of isoflavones can cause changes in sex hormone production and perturb menstruation. The recently published data (Jefferson *et al.* 2005) indicate a potential deleterious role of isoflavones during pregnancy, which is of particular concern. The authors reported that neonatal exposure to genistein at environmentally relevant doses caused abnormal oestrous cycles, altered ovarian function, early reproductive senescence, and subfertility in mice. Whether TTR itself or altered thyroid hormone economy as suggested by the present observations contribute to alterations of the reproductive axis reported by Jefferson *et al.* (2005) remains to be studied.

Competitive *in vitro* binding with TTR in CSF revealed a clear increase in free T_4 concentration because there is no other alternative specific thyroid hormone carrier able to bind displaced T_4. TBG is not present in CSF (Davidsson *et al.* 2001; Matsumoto *et al.* 2003) and the amount of albumin which has the lowest affinity to bind T_4 (Tabachnick & Giorgio, 1964) is too low compared with TTR to bind displaced [^{125}I]T_4. Chanoine *et al.* (1992) also reported the transient increase in serum and CSF free T_4 concentration after administering low and high doses of synthetic flavonoid EMD21388, respectively. However, the occupation of the sole hormone distributor protein and evident disturbance in binding properties postulate an effect on brain metabolism, if it is known that thyroid hormones are intimately involved in the regulation of the central nervous system. Experimental studies show that the central nervous system has strict requirements for thyroid hormones; in the brain, the concentrations of both T_4 and the more active metabolite triiodothyronine tend to be kept within a narrow range even in the presence of extreme fluctuations of circulating T_4 level (Dratman *et al.* 1983). This fact additionally underlines the importance of TTR binding in CSF considering that TTR binds both T_4 and triiodothyronine (although having different affinities) (Cody, 2002), which are present in equimolar concentration in the brain in contrast to the serum. However, further *in vivo* studies are required to clarify the physiological and molecular implications of disturbed T_4 binding, particularly on the central nervous system.

The negative influence of soya isoflavones on thyroid hormone synthesis by means of blocking thyroid peroxidase has been well described *in vitro* and *in vivo*. Numerous studies on rats and human subjects raise concerns on anti-thyroid effects, including goitre formation, especially in infants consuming soya formula (van Wyk *et al.* 1959; Hydovitz, 1960; Shepard *et al.* 1960; Ripp, 1961; Pinchera *et al.* 1965; Kay *et al.* 1988; Labib *et al.* 1989; Ishizuki *et al.* 1991; Chorazy *et al.* 1995; Jabbar *et al.* 1997; Ikeda *et al.* 2000). There are also opposite reports (Klein, 1998; Merritt & Jenks, 2004) indicating that dietary isoflavones in soya infant formulas do not adversely affect human health. Besides the inhibitory effects of flavonoids on thyroid peroxidase, iodine deficiency is a very important risk factor for thyroid dysfunction and goitre development in both man and rats. An adequate iodine supply is absolutely advantageous for preventing the goitrogenic effects of soya isoflavones, especially in the relatively high-risk group of patients with congenital hypothyroidism solely dependent on exogenous thyroid hormone supply, or in patients with transient hypothyroidism after thyroidectomy. The present data contribute to unveiling the mechanism of action of soya isoflavones in goitrogenesis and disturbance

of the thyroid hormone homeostasis, while emphasising the role of binding and distributor proteins, particularly TTR.

TTR is the main thyroid hormone transport protein in rodents, but, in man, despite the 20-fold higher concentration in serum relative to that of TBG, it plays a lesser role in iodothyronine transport (Woeber & Ingbar, 1968). Only a minor fraction of T_4 is bound to serum albumin in both man and rats, despite the very high binding capacity (Köhrle *et al.* 1989). *In vitro* and *in vivo* competitive-binding studies have revealed up to now that flavonoids interfere with T_4 binding, not with all thyroid hormone distribution proteins, but preferentially with TTR (Köhrle, 2000). TTR is a highly conserved tetrameric protein with two binding domains and three pairs of halogen-binding pockets in each of them (Cody, 2002). At physiological conditions, only one T_4 binding domain is occupied, since the negative cooperativity in binding to the second domain decreases the binding affinity of a second hormone molecule (Ferguson *et al.* 1975). Structural data for the human TTR–T_4 complex has revealed that T_4 binds in a 'forward' mode with its phenolic OH group buried deep within the binding channel, while the synthetic flavone EMD21388 binds to human TTR in a manner different from T_4 (Cody, 2002). After 12 h incubation with EMD21388, binding occurred only in one domain in a 'forward' mode, while 24 h incubation data showed forward binding in both domains with the bromoflavone bound deeper in the channel than T_4. After 48 h incubation EMD21388 binds in both a 'forward' mode and a 'reverse' mode. Ciszak *et al.* (1992) reported the similar binding manner for a bromoaurone analogue. The fact that soya isoflavones tested in the present study have a similar structure to synthetic bromoflavone means that a similar binding manner could be expected. Therefore, soya isoflavones could also exhibit alternative binding orientations, which may explain such strong binding affinities for TTR (Auf'mkolk *et al.* 1986). Structure–activity correlation in the present study revealed the soya isoflavone genistein as the strongest binding competitor, indicating the same binding potency as L-T_4. Obviously, the 5-OH group is important for binding, most probably by occupying one of the three halogen-binding pockets, while the 7-OH group in the meta-position occupies the other. In addition, the ability of the 5-OH group of genistein to form an intramolecular hydrogen bond with the 4-keto group leading to a pseudo aromatic ring (Chen *et al.* 1995) could also participate in increased binding to TTR compared with other soya isoflavones. The reason for the ten-fold increase in binding potency of glycitein compared with daidzein should be searched for in the 6-methoxy group which is absent in daidzein. According to Cody (2002), the presence of the 3-methyl of EMD21388 appears crucial for effective binding and stabilisation of the TTR tetramer. The important van der Waal's interactions may also be formed between the 6-methoxy group of daidzein and adequate amino acids in the binding pocket. However, the further crystallographic analyses of TTR–ligand co-crystal complexes are required to clarify these structure–activity relationships.

Naturally occurring chalcones, including phloretin, aurones and flavonoids, exhibit a clear concentration-dependent displacement of T_4 from TTR binding *in vitro* with IC_{50} values in the range of 0·1–50 μM (Köhrle, 2000). The present data contribute to competition-binding studies made thus far, therefore revealing the isoflavone genistein as the strongest naturally occurring T_4 competitor. Further *in vivo* studies will clarify the expected disturbance in the feedback regulation of hormonal networks, including the pituitary–thyroid–periphery axis and will shed more light on the role of TTR in this feedback circuit.

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