RESEARCH ARTICLE

Synthetic isoflavones and doping: A novel class of aromatase inhibitors?

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Abstract
Anecdotal information suggests that flavonoids may be widely used among athletes for their multiple biochemical and pharmacological effects. We have evaluated in vitro the effects of two synthetic isoflavones, methoxyisoflavone and ipriflavone, on the catalytic activity of human aromatase (CYP19), the enzyme catalyzing the conversion of androgens (ie, testosterone or androstenedione) to estrogens (ie, estradiol and estrone). The potential inhibitory effect was evaluated by measuring the rate of aromatization of testosterone, monitored by gas chromatography–mass spectrometry (GC–MS), both in the presence and in the absence of methoxyisoflavone or ipriflavone, comparing their effects with those of synthetic aromatase inhibitors (formestane, anastrozole, and aminoglutethimide) presently included in the list of prohibited substances and methods, and of natural flavonoids (chrysin, quercetin, and daidzein), that are known inhibitors of CYP19. The preliminary results of our in vitro study show that methoxyisoflavone and ipriflavone act as competitive inhibitors of aromatase, the degree of inhibition measured in vitro being of the same order of magnitude of that of the aromatase inhibitors commonly used in anti-estrogenic therapies. Our preliminary in vitro results indicate that, in principle, a sufficiently large intake of isoflavones could alter the kinetics of the dynamic equilibria between androgens and estrogens, suggesting their monitoring in doping control routine analysis.

KEYWORDS
doping, synthetic flavonoids, ipriflavone, methoxyisoflavone, aromatase

1 | INTRODUCTION
Flavonoids are a broad class of low molecular weight compounds widely found in plants and fruit. They occur in common human diet and have been used as ingredients of drugs or food supplements.1,2 From a chemical point of view, flavonoids are characterized by a basic chemical structure consisting of two benzyl rings (A and B) joined by a three-carbon bridge which may or may not closed in a pyran ring (Figure 1A). According to the position of B ring and to the level of unsaturation and oxidation of C ring, flavonoids can be divided in different groups, including but not limited to flavones, isoflavones, flavonols, anthocyanins, and catechins.

Flavonoids have been used as anti-oxidants, anti-estrogenic agents, and androgen promoters. They have been shown to be endowed with many structure-dependent activities, leading to cytostatic, apoptotic, anti-inflammatory, anti-angiogenic, and hepatoprotective effects.3,4 and attracting attention also as possible chemoprotective or chemotherapeutic agents.5 In addition, several flavonoids are modulator of expressions and activity of specific cytochrome P450 genes and/or proteins.6 Nevertheless, due to their low concentration in food and to their non-optimal oral bioavailability, the actual effects of dietary flavonoids are moderately low.7 Among them, isoflavones, often classified as “phytoestrogens” due to their weak estrogenic activity, may be of interest in sports doping for their
isoflavones are part of a group of substances of plant origin called 
estrogenic or/antioxidant properties.\textsuperscript{8,9} The isoflavones structure 
differs from the isomeric flavones only for the position of phenyl B 
ring (Figure 1B). The consumption of food containing a large amount of 
flavonoids seems to be adjuvant for the course of cardiovascular 
diseases, cholesterolemia, premenstrual syndrome, menopause, and 
osteoporosis.\textsuperscript{10,11} For their medical and therapeutic interest, the most 
studied natural isoflavones are daidzein, genistein, and glycitein.\textsuperscript{12,13}

Based on the biopharmacological properties of their natural ana-
logs, synthetic isoflavones have been designed, synthesized, and stud-
yed for their biological effects. Among them, special attention has 
been focused on methoxyisoflavone (5-methoxy-7-methoxy-isoflavone) 
(Figure 1C) and ipriflavone (7-isopropoxy-isoflavone) (Figure 1D).\textsuperscript{8}

In the 1970s, animal studies showed the anabolic properties of 
methodoxyisoflavone in terms of increased protein synthesis and 
decreased cortisol blood level.\textsuperscript{14} Human studies did not show signifi-
cant modification of some key parameters usually considered in the 
assessment of anabolic properties (among them, body weight, body 
mass index, blood cortisol level, and isokinetic peak force) and for this 
reason the anabolic and ergogenic properties of methoxyisoflavone 
have not been further explored.\textsuperscript{15,16} Furthermore, focus on 
methodoxyisoflavone in forensic toxicological analysis has been limited to 
the fact that its metabolites can cross-react with the antibodies 
used for the screening of synthetic cannabinoids, causing false-
positive results in urinary immunoassay.\textsuperscript{17}

The use of ipriflavone is indicated in the treatment of osteoporosis 
in postmenopausal women\textsuperscript{18} due to its normalizing effects on calcito-
nin levels. Ipriflavone and its most abundant metabolite, 7-hydroxy-isof-
lavone, can also inhibit CYP1A2 and CYP2C9, leading to an increase in 
plasma levels of some drugs, including theophylline and tolbutamide.\textsuperscript{19}

As for their effects on the estrogen metabolism and receptors, 
isoflavones are part of a group of substances of plant origin called 
"phytoestrogens" or "weak estrogens," due to the modulatory effects 
that exert at level of estrogen receptors, pregnane X receptors, and 
constitutive androstane receptors.\textsuperscript{20-22} Although with an affinity 
approximately 5000 times lower than that of the estrogens,\textsuperscript{23} 
isoflavones can act as partial agonists or antagonists of the estrogen 
receptors, most probably through the inhibition of aromatase 
(CYP19)\textsuperscript{24-26} that is the enzyme that catalyzes the conversion of 
androstenedione and testosterone to estrone and estradiol 
respectively.\textsuperscript{27} The estrogenic/antiestrogenic activity of isoflavones 
may also be due to an alteration of biosynthesis and subsequent 
secretion of luteinizing hormone (LH), that acts stimulating the pro-
duction of testosterone in male testicles, and that it is therefore 
cluded in the Prohibited List of the World Anti-Doping Agency 
(WADA), in section S2 (Peptide hormones, growth factors, related 
substances and mimetics),\textsuperscript{28} while isoflavones are non-prohibited 
substances.

The goal of the present work is to evaluate the effects of the two 
main synthetic isoflavones, methoxyisoflavone and ipriflavone, on the 
catalytic activity of the enzyme human aromatase (CYP19) by in vitro 
hibition kinetics experiments monitored by gas chromatography-
mass spectrometry (GC-MS), comparing the entity of the effects with 
those produced by known inhibitors of aromatase, namely (a) natural 
flavanoids (chrysin, quercetin and daidzein),\textsuperscript{29-31} and (b) synthetic 
aromatase inhibitors already considered in the WADA Prohibited List 
(formestane, anastrozole, and aminoglutethimide, included in section 
S4 of Hormones and metabolic modulators).

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Testosterone, estradiol, formestane, aminoglutethimide, daidzein and 
quercetin were from Sigma-Aldrich (Milan, Italy); testosterone-\textsuperscript{252} 
(used as internal standard) was from the National Measurement 
Institute (NMI, Pymble, Australia); anastrozole was from TRC Canada 
(Toronto, Canada); chrysin was from Honeywell Fluka (Rodano, 
Italy); methoxyisoflavone and ipriflavone were supplied by LGC 
Standards (Sesto San Giovanni, Italy). The derivatizing agent was a 
mixture of N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) / 
mercaptopoethanol/ammonium iodide (NH\textsubscript{4}I) (1000:6:4 v/v/v) stored in 
screw-cap vials at 4°C for a maximum of two weeks. MSTFA was 
 supplied by Chemische Fabrik Karl Buecher GmbH (Walidetten, 
Germany); NH\textsubscript{4}I and mercaptopoethanol were from Sigma-Aldrich 
(Milan, Italy). Solvents (tert-butylmethyl ether, ethyl acetate and 
 methanol) and reagents (sodium phosphate and sodium hydrogen 
phosphate) were of analytical or high-performance liquid chromatog-
raphy (HPLC) grade and provided by Sigma-Aldrich (Milan, Italy).

Water was obtained from a MilliQ water purification system (Millipore 
S.p.A., Milan, Italy). The reagents (sodium phosphate buffer and the 
NADPH regenerating system consisting of magnesium chloride hexa-
hydrate, NADP\textsuperscript{+}, glucose-6-phosphate and glucose-6-phosphate 
dehydrogenase) for the in vitro study were purchased from GenTest 
(Tewksbury, MA, USA). Human aromatase (CYP19 + P450 reductase) 
was from Discovery Labware Inc (Woburn, MA, USA).

2.2 | In vitro protocol

Working solutions of testosterone (0.1, 0.2, 0.5, 1, 5, 10, 20 \textmu M) were 
prepared from a 50 \textmu M stock solution in methanol. Working solutions of 
inhibitors, prohibited drugs, and natural flavonoids, were prepared at 
different concentration, according to their IC\textsubscript{25}, IC\textsubscript{50} and IC\textsubscript{75} 
(formestane, anastrozole, and aminoglutethimide 0.4, 0.8, 1.2 \textmu M; 
daidzein 1, 5, 10 \textmu M; chrysin 0.35, 0.7, 1 \textmu M; quercetin 25, 50,
100 μM), in methanol.29-31 Stock solution of methoxyisoflavone and ipriflavone (5, 10, 50 μM) were prepared in methanol. The incubation mixture, in a final volume of 150 μL, contained 0.1 M sodium phosphate buffer, testosterone at final concentration of 1, 5, 10, 20, 50 μM, an NADPH regenerating system consisting of 3.3mM magnesium chloride, 1.3mM NADP+, 3.3mM glucose 6-phosphate and 0.4 U/mL glucose-6-phosphate dehydrogenase. After mixture pre-warming at 37°C for 5 minutes, the reaction, either in the absence or in the presence of different concentrations of the inhibitor, was started by adding 1 μM of human aromatase. The samples were then incubated at 37°C up to 1440 minutes. One sample (negative control) containing all reaction components, but without the enzymatic protein, was also processed to monitor the potential non-enzymatic reactions possibly taking place during the incubation period. The overall reaction was terminated by the addition of 150 μL of ice-cold acetonitrile. The reaction mixtures were then centrifuged (12 000 rpm for 7 minutes) before the sample purification. All incubations were performed in triplicate.

2.3 Sample preparation

The supernatant of the samples obtained by the in vitro experiments were, after centrifugation, brought to dryness. 1 mL of phosphate buffer 0.8 M (pH 7.4) and 100 μL of the internal standard (testosterone-d₃ 0.5 μM) were added; the liquid/liquid extraction was carried out with 5 mL of tert-buthylmethyl ether for 5 minutes on a mechanical shaker. After centrifugation the organic layer was transferred to a 10 mL tube and evaporated to dryness. The residue was reconstituted in 50 μL of the derivatizing mixture and the sample was maintained at 75°C for 30 minutes. Then a 1 μL aliquot was injected into the GC–MS system.

2.4 Instrumental analysis

Instrumental analysis was performed on an Agilent 6890/5973A gas chromatography–mass spectrometry (GC–MS) system (Agilent Technologies, Milan, Italy), in electron ionization (70 eV) mode, using an HP-1 fused silica capillary column (cross-linked methyl silicone, length 17 m, i.d. 0.20 mm, film thickness 0.11 μm; Agilent Technologies, Milan, Italy). The GC condition were as follows: the carrier gas helium at a flow rate of 1 mL/min; split ratio of 1/10; temperature program, 180°C (4.5-minute hold), 3°C/min to 230°C, 20°C/min to 290°C and 30°C/min to 320°C; the transfer line and injection temperature operated at 280°C. The acquisition was performed in selected ion monitoring (SIM) mode. Identification was based on the specific retention time and on the characteristic diagnostic ions listed in Table 1. Quantitative determination of the concentration of testosterone and estradiol after enzymatic reaction was based on the peak area ratio of the analyte to the internal standard (testosterone-d₃).

### TABLE 1 Retention time and characteristic ions for testosterone (T), estradiol (E₂) and testosterone-d₃ (T-d₃)

<table>
<thead>
<tr>
<th></th>
<th>Retention Time (min)</th>
<th>Diagnostic Ion (m/z) 1</th>
<th>Diagnostic Ion (m/z) 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (T)</td>
<td>14.11</td>
<td>432</td>
<td>417</td>
</tr>
<tr>
<td>Estradiol (E₂)</td>
<td>13.93</td>
<td>416</td>
<td>285</td>
</tr>
<tr>
<td>Testosterone-d₃ (T-d₃)</td>
<td>14.08</td>
<td>435</td>
<td>420</td>
</tr>
</tbody>
</table>

2.5 Data analysis

For peak integration and analyte quantification, a proprietary software of the GC–MS system was used. Peak integration was always double-checked manually.

Michaelis–Menten’s kinetic constant (Kₘ) and the maximum velocity of reaction (Vₘₐₓ) of the biotransformation of testosterone in estradiol were evaluated by nonlinear regression analysis carried out on the Lineweaver–Burk plot of the biotransformation of testosterone (T) in estradiol (E₂) [Colour figure can be viewed at wileyonlinelibrary.com]
out using GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA). The inhibition constant's value (K_i) of the biotransformation of testosterone to estradiol in the presence of different concentration of methoxyisoflavone and ipriflavone (5, 10, 50 μM) was evaluated through graphical analysis of Lineweaver-Burk plot and by fitting data into competitive, uncompetitive, non-competitive and mixed inhibition models by nonlinear regression analysis carried out using GraphPad Prism version 7.0.

3 | RESULTS AND DISCUSSION

3.1 | In vitro biotransformation of testosterone to estradiol in the presence of CYP19

To preliminarily verify the reliability of the data obtained in our in vitro metabolism studies, the biotransformation of testosterone to estradiol was preliminarily followed in the absence of inhibitors. As expected, a

FIGURE 3 Concentration of substrate (E2) vs time plot of the biotransformation of testosterone in estradiol without and the in the presence of A, formestane, B, anastrozole, and C, aminoglutethimide in three different concentrations (μM) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 4 Concentration of substrate (E2) vs time plot of the biotransformation of testosterone in estradiol without and in the presence of A, chrysin, B, quercetin, and C, daidzein in two (daidzein) and three different concentrations (μM) [Colour figure can be viewed at wileyonlinelibrary.com]
decrease in testosterone concentration and a parallel increase of estradiol concentration were observed. To calculate the kinetic parameters of the in vitro biotransformation of testosterone to estradiol the substrate was incubated, at different concentration (1, 5, 10, 20, 50 µM), for 1440 minutes. Michalis-Menten plot, Lineweaver-Burk plot, $K_m$ (1.43 µM) and $V_{max}$ (0.24mM s$^{-1}$) were calculated.

**TABLE 2** Inhibition kinetic constant ($K_i$) and type of inhibition for all compounds studied

<table>
<thead>
<tr>
<th>Aromatase inhibitor drugs</th>
<th>$K_i$ (µM)</th>
<th>Inhibition Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formestane</td>
<td>0.20 $&lt; K_i &lt; 0.65$</td>
<td>Competitive suicide</td>
</tr>
<tr>
<td>Anastrozole</td>
<td></td>
<td>Competitive reversible</td>
</tr>
<tr>
<td>Aminoglutethimide</td>
<td></td>
<td>Competitive reversible</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Natural flavonoids</th>
<th>$K_i$ (µM)</th>
<th>Inhibition Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysin</td>
<td>2.20 $&lt; K_i &lt; 2.80$</td>
<td>Competitive</td>
</tr>
<tr>
<td>Quercetin</td>
<td></td>
<td>Competitive</td>
</tr>
<tr>
<td>Daidzein</td>
<td></td>
<td>Competitive</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Synthetic isoflavones</th>
<th>$K_i$ (µM)</th>
<th>Inhibition Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxyisoflavone</td>
<td>0.36</td>
<td>Competitive</td>
</tr>
<tr>
<td>Ipriflavone</td>
<td>0.19</td>
<td>Competitive</td>
</tr>
</tbody>
</table>

**FIGURE 5** Concentration of substrate (E2) vs time plot of the biotransformation of testosterone in estradiol without and in presence of A, methoxyisoflavone and D, ipriflavone in three different concentrations (µM); Michaelis–Menten plot and Lineweaver plot of the biotransformation of testosterone in estradiol in presence, respectively, of three different concentration (µM) of B, C, methoxyisoflavone and E, F, ipriflavone [Colour figure can be viewed at wileyonlinelibrary.com]
obtained by non-linear regression analysis. The low value of $K_i$ is related to the remarkable affinity of testosterone with human aromatase, in our in vitro model (Figure 2A–C).

3.2 | In vitro inhibition of CYP19 by formestane, anastrozole, and aminoglutethimide

Successively, to validate our in vitro model systems also for the study of CYP19 inhibition, we evaluated the effects of the synthetic aromatase inhibitors formestane, anastrozole, and aminoglutethimide, on the in vitro biotransformation of testosterone to estradiol. More specifically, formestane is a steroidal, competitive, and irreversible inhibitor of human aromatase, while anastrozole and aminoglutethimide are non-steroidal, competitive and reversible inhibitors. These drugs are clinically used to treat postmenopausal breast cancer and other estrogen-dependent disease. After the incubation of different concentrations (0.4, 0.8, and 1.2 μM) of each drugs with 1 μM of substrate (testosterone), a pronounced reduction of estradiol concentration was observed, this effect being independent of the concentration of banned drug. These results confirmed that formestane, anastrozole, and aminoglutethimide resulted as competitive inhibitors of CYP19 also in our in vitro model system (Figure 3A–C).

3.3 | In vitro inhibition of CYP19 by chrysin, quercetin and daidzein

Furthermore, the same in vitro assays were also performed in the presence of chrysin, quercetin, and daidzein. Chrysin and quercetin are natural flavones while daidzein is a natural isoflavone. Despite their in vitro inhibition effects on the catalytic activity of aromatase is well-documented, these substances are not banned or subject to monitoring by WADA. After incubation at different concentrations, in the previously described experimental conditions, the inhibitory effect was assessed to be concentration-independent for chrysin and quercetin, and concentration-dependent for daidzein. (Figure 4A–C).

3.4 | In vitro inhibition of CYP19 by ipriflavone and methoxyisoflavone

Having therefore preliminarily verified the reliability of the results obtained in our in vitro model system, we proceeded with the assessment, in the same experimental conditions, of the potential effects of ipriflavone and methoxyisoflavone on the catalytic activity of aromatase. The in vitro biotransformation of testosterone to estradiol catalyzed by CYP19 was studied in the presence of different concentrations of either one of the two synthetic isoflavones (methoxyisoflavone and ipriflavone) specifically considered in this study. After the incubation at different concentrations (5, 10, 50 μM), a decrease of the rate of formation of estradiol was recorded, this effect being concentration-dependent for methoxyisoflavone and concentration-independent for ipriflavone. To evaluate the kinetic parameters of the in vitro biotransformation of testosterone in estradiol, the substrate (testosterone) was incubated, at different concentrations (1, 5, 10, 20, 50 μM), for 1440 minutes with different concentrations of methoxyisoflavone or ipriflavone at 5, 10, and 50 μM. The results obtained after a non-linear regression analysis show that $K_i$ value for methoxyisoflavone is 0.36 μM and for ipriflavone is 0.19 μM. Michaelis–Menten plot and Lineweaver-Burk plot show that methoxyisoflavone and ipriflavone inhibit in a competitive manner human aromatase (Figure 5A–F).

The $K_i$ values obtained for synthetic isoflavones are of the same order of magnitude of those of banned drugs, and significantly smaller than those of natural flavones considered in the study (Table 2).

3.5 | Conclusions

The results obtained in our in vitro model system show that, in the range of concentrations here considered, methoxyisoflavone, and ipriflavone have a potential for aromatase inhibition similar to that of formestane, anastrozole, and aminoglutethimide, that are drugs used in anticancer therapy and banned by the WADA for their effects on steroidogenesis. Clearly, the results here presented are not sufficient to express a final opinion on the opportunity of including synthetic isoflavones on WADA’s Prohibited List, since the actual magnitude of their in vivo effects would certainly be modulated by their pharmacokinetic properties, and especially by their low bioavailability. Nonetheless, we believe their monitoring could still be useful in doping control analysis, also considering the high dosages recommended for synthetic isoflavones in dietary supplements. For indeed, regardless the in vivo pharmacokinetic effects, the intake of synthetic isoflavones, especially in association with prohibited androgens, their precursors and/or metabolites, could alter the kinetics of the dynamic equilibria between androgens and estrogens, also potentially affecting some parameters of the steroid module of the athlete biological passport (ABP).

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