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Review

Synthetic derivatives of genistein, their properties and possible applications

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Genistein, the principal isoflavone constituent of soybean, attracts much attention as a natural molecule with significant affinity towards targets of potential medicinal interest, but also as a food supplement or prospective chemopreventive agent. Since its physicochemical properties are considered suboptimal for drug development, much effort has been invested in designing its analogs and conjugates in hope to obtain compounds with improved efficacy and selectivity. The aim of this article is to summarize current knowledge about the properties of synthetic genistein derivatives and to discuss possible clinical application of selected novel compounds. Some basic information concerning chemical reactivity of genistein, relevant to the synthesis of its derivatives, is also presented.

Keywords: synthetic genistein derivatives, inhibition of cell proliferation, tyrosine kinases, selective estrogen receptor modulators, anticancer activity, antimicrobial activity

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INTRODUCTION

Ethnopharmacological tradition followed by pharmacognosy and backed up by modern analytical techniques have provided numerous examples of plant-derived compounds which exhibit selective toxicity or otherwise distinct biological activity. Secondary plant metabolites not only provided the foundation of folk medicine and generations of traditional drugs, but also continue to be an inspiration for studies towards modern medicinal applications (Reuben *et al.*, 2005; Dewick, 2009).

Isoflavones constitute a sub-class of flavonoids, a large family of secondary plant metabolites, which share common structural feature: a C6–C3–C6 sequence of the carbon skeleton, split into several variants of heterocyclic ring substitution pattern and diversified by a plethora of further modifications (Dixon, 1999; Wiseman, 2006; Veitch, 2007).

The role of isoflavones for the plant is not entirely clear and their target proteins are not known. It is likely that their formation has constitutive, as well as inducible determinants (Buer *et al.*, 2007). Isoflavones are considered phytoalexins, which can also offer some marginal environmental advantage through allelopathic interactions. They possess antimicrobial and anti-insect activity, and induce nodulation genes in symbiotic *Rhizobium* bacteria. Nevertheless, it has to be stressed that the occurrence of isoflavones is quite limited in comparison with other types of flavonoids, like flavones, flavonols, anthocyanins, aurones, coumestans, etc. Plants known for significant isoflavone content are mainly found in the family of *Fabaceae*.

Water solubility of isoflavonoids is extremely low and their trafficking within a plant requires glycosylation, performed by ubiquitous glycosyltransferases (Offen *et al.*, 2006; Noguchi *et al.*, 2007).

Genistein (**1**), (Fig. 1) one of the most extensively studied isoflavones, as well as its analogs, are generally recognized as phytoestrogens. In fact, they can exert estrogenic as well as anti-estrogenic action, which is sometimes connected with animal reproduction problems (Leopold *et al.*, 1976; Jefferson *et al.*, 2007; Eustache *et al.*, 2009).

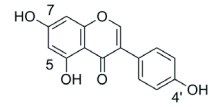

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Figure 1. Genistein

The interest in genistein, as a potential chemopreventive agent or a drug supplementing treatment of relevant diseases arose with the discovery of estrogenic properties of isoflavonoids. This property suggests genistein use as a diet supplement relieving side effects of estrogen deficiency in menopausal women. On the other hand, the discovery of cytotoxic and antiproliferative activity of genistein, *via* inhibition of tyrosine kinases (Akiyama *et al.*, 1987) and topoisomerase II (Markovits *et al.*, 1989), as well as G2/M block of the cell cycle (Matsukawa *et al.*, 1993) has suggested possible application of genistein in anticancer therapy.

Anticancer properties of genistein deduced from the results of *in vitro* experiments have been supported by epidemiological observations, indicating that increased soy consumption is related to lower risk of cancer development and cancer-related death especially in the case of breast and prostate cancer (Adlercreutz *et al.*, 1993; Morton *et al.*, 1997; Hussain *et al.*, 2002; Magee & Rowland,

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Abbreviations: ALL, acute lymphoblastic leukemia; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, estrogen receptor; GAGs, glycosaminoglycans; LDL, low density lipoprotein; MPs, mucopolysaccharidoses; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ox-LDL, oxidized low density lipoprotein; SERM, selective estrogen receptor modulator; UDPG, uridine diphosphoglucose.

2004; Holzbeierlein *et al.*, 2005; Chan *et al.*, 2005; Sonn *et al.*, 2005).

Although genistein exhibits biological activity of potential medicinal interest, there are also some drawbacks to be considered, limiting its prospective application in clinical practice. Among the most important are: relatively low concentration in the blood following *per os* application, due to relatively poor absorption from the intestine, rapid biotransformation *in vivo* into inactive metabolites, and ineffective accumulation in cells and tissues (Dixon & Ferreira, 2000; Shellnut *et al.,* 2002; Simons *et al.,* 2010). At least some of these unfavourable features are believed to be diminished by chemical modification of genistein. But it is also expected that some genistein derivatives could exhibit increased selectivity to known molecular targets or acquire activity directed against novel ones.

The biological and biochemical activity of genistein, its potential chemopreventive and therapeutic properties, as well as genistein affinity towards a large variety of molecular targets have been reviewed in several excellent articles (Sarkar *et al.*, 2006; Banerjee *et al.*, 2008; Meeran & Katiyai, 2008; Sakai & Kogiso, 2008). The aim of this article is to present current findings concerning possible clinical applications of selected novel synthetic derivatives of genistein and some basic information on chemical reactivity of genistein, relevant to the synthesis of its derivatives.

Among many arguments cited in favour of synthetic analogs of genistein as prospective new drug candidates, in our opinion two are particularly convincing:

- the natural pool of the isoflavone consists of three different types of chemical entities — the aglycone, glucoside and acylated glucosides, which apparently facilitates trafficking and compartmentalization in plant tissue and may have a profound effect on the bioavailability and pharmacokinetics in humans;
- genistein, even though practically insoluble in water in its basic form and poorly bioavailable as aglycon, exhibits pleiotropic biological activity. This indicates the existence of multiple molecular targets, and an obvious potential of tuning up the drug's affinity by its derivatization.

SEARCH FOR GENISTEIN DERIVATIVES EXHIBITING INCREASED INHIBITORY ACTION AGAINST TYROSINE KINASE

One of the early observations relevant to possible anticancer applications of genistein concerned the inhibition of tyrosine kinases, including c-Src and v-Abl (Akiyama *et al.*, 1987). These enzymes catalyze selective transfer of γ-phosphate groups from ATP to tyrosine residues of proteins, and this phosphorylation is important for controlling various signal transduction pathways involved in proliferation, differentiation, cell migration and many other cellular activities. Increased or aberrant expression or tyrosine kinases is regarded as one of important factors influencing tumour development and progression (Manash & Mukhopadhyay, 2004; Shchemelinin *et al.*, 2006).

The major structural features of genistein relevant to its inhibitory action against tyrosine kinase have been established by comparing the inhibitory effectiveness of several isoflavones with similar structure (daidzein, flavone, biochanin A, acacetin, prunetin, genistin). The rate of phosphorylation of A431 cell membranes induced by epidermal growth factor (EGF) in the presence of the above isoflavones indicated that critical for the inhibitory action of genistein is the OH group at C-5, and that the presence of OH groups at the C-7 and C-4' positions is responsible for the highest inhibitory activity of genistein (Ogawara *et al.*, 1989). Importantly, a bulky group at C-7 was found to abolish the inhibitory action of genistein.

Having in mind that substitution of the OH groups at C-4', C-5, and C-7 can interfere with the inhibitory activity of genistein, Ogawara *et al.* (1989) synthesized 23 derivatives of isoflavone with various groups attached to C-2. Each of those derivatives was tested for its cytotoxicity (IC_{50}) as well as the ability to inhibit tyrosine kinase activity. It was found that none of the tested drugs was more cytotoxic than genistein and none exhibited a greater anti-kinase activity.

Booth *et al.* (1999) synthesized a series of compounds with different pattern of methoxylation and oxygenation on the phenyl ring. However, none of those derivatives exhibited increased cytotoxic activity against colon cancer cells, and only one of them inhibited tyrosine kinase activity, albeit less efficiently than genistein. To our knowledge, the search for genistein derivatives able to inhibit tyrosine kinase activity more efficiently than genistein has turned out to be unsuccessful so far.

GENISTEIN DERIVATIVES WITH THE ACTIVITY OF SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMS)

It was recognized very early that the chemical structure of genistein bears strong similarity to 17β-estradiol, and that genistein binds to estrogen receptors (ER) and to sex hormone-binding globulins (Kuiper *et al.*, 1998; Klinge, 2000; Kurzer, 2002). A functional interaction of genistein with estrogen receptors, leading to stimulation of ER responsive genes, was confirmed in multiple experiments *in vitro* (Birt *et al.*, 2001; Kostelac *et al.*, 2003; Zierau *et al.*, 2006). Although genistein interacts with both ERα and ERβ, it shows a much higher affinity (approx. 30 times higher) to ERβ (Pike *et al.*, 1999). Moreover, the similarity of the mode of interaction between estrogen receptor and genistein to that of receptor and raloxifen (Pike *et al.*, 1999) reasonably suggests that genistein should be classified as one of natural selective estrogen receptor modulators (SERMs) and not as an estrogen (Setchell, 2001). To some extent, genistein action resembles the effects of raloxifen on the skeletal system in ovariectomized mice (Śliwiński *et al.*, 2009).

In order to increase the SERM properties of genistein a number of genistein derivatives have been synthesized and their binding to ER receptors assessed. One of them, 6-carboxymethyl genistein (6CG) (Fig. 2) (Somjen *et al.*, 2002) was found to bind highly selectively to ERβ, although the binding affinity was smaller than that of genistein. Interestingly, genistein and 6CG exhibited differential influence on DNA synthesis in human umbilical artery vascular smooth muscle cells. While low concentration of genistein (30 nM) increased DNA synthesis induced by low concentrations of estrogen (0.3 nM), an equivalent dose of 6CG had an inhibitory effect.

To find out whether the antiosteoporetic properties of genistein could be enhanced by its modification, Wang *et al.* (2005) synthesized, with the use of a sonochemical method, a number of genistein derivatives in which the C-7 or C-4' hydroxyl groups were substituted with various chemical groups (Fig. 3).

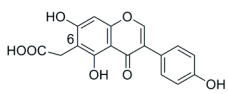

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Figure 2. 6-Carboxymethylgenistein
According to Somjen *et al.* (2002).

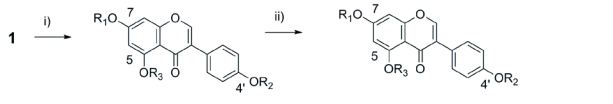
Those novel genistein derivatives, except the toxic ones, were subsequently administered to ovariectomized rats in which experimental osteoporosis had been induced by feeding the animals a low-calcium diet. The compounds, including genistein, were administered orally through a stomach tube at a dose of 25 μmol/kg per day. As a positive control, anti-17β-estradiol was used at 0.5 μmol/kg per day. Two months later measurements of the femoral bone mineral density, and the level of alkaline phosphatase, a bone metabolic marker, were conducted.

Among seventeen novel genistein derivatives the authors found five compounds showing increased antiosteoporetic activity when compared to genistein. The best results were observed for 4',5,7-tri[3-(2-hydroxyetylthio) propoxy]isoflavone. All bioactive compounds showed no acute toxicity; they contained the 2-hydroxyetylthio motif, which the authors assumed to be a key pharmacophore, inhibiting bone loss during estrogen shortage.

MODIFICATION OF GENISTEIN AIMED TO IMPROVE OR CHANGE ITS BIODISTRIBUTION

Conjugation of genistein with antibodies or peptide ligands

One of the major strategies of targeted therapy is to construct two-domain drugs in which one domain recognizes the target cells, whereas the other one exerts a therapeutic activity. In order to selectively target genistein to transmembrane tyrosine kinases with their kinase domain localized intracellularly, Uckun *et al.* (1998) obtained *via* photochemical cross-linking a conjugate of genistein with epidermal growth factor (EGF), an epidermal growth factor receptor (EGFR) ligand. It was expected that internalization of this conjugate should increase the intracellular concentration of genistein leading to a more effective inhibition of the EGFR tyrosine kinase activity. Subsequent biodistribution studies in mice revealed that, as expected, the conjugated genistein was effectively taken up by cells, and the highest concentration of the conjugate was observed



i) RBr,K_2CO_3, sonification; ii) **3** or **4**, HO-CH_2CH_2-SH

7-mono-, (R_1=R, R_2=R_3=H); 7,4' di-, (R_1= R_2, R_3=H); 5,7,4'- trisubstituted derivatives, (R_1=R_2=R_3=R)

3 R= Br-$(CH_2)_n$- ; n=2,

4 R= Br-$(CH_2)_n$- ; n=3

5 R= EtO_2C-CH_2-

6 R= HO-CH_2-CH_2-S-CH_2-CH_2-

7 R= HO-CH_2-CH_2-S-CH_2-CH_2-CH_2-

Figure 3. Derivatives of genistein with antiosteoporotic properties
According to Wang *et al.* (2005).

in the liver and spleen. Unexpectedly, the authors observed low accumulation rate of the conjugate in human breast cancer cells (line MDA-MB-231) grown on SCID mice. However, mice treated intraperitoneally with the conjugate at 100 μg/day for 10 days showed significantly better survival as compared to mice treated with adriamycin, cyclophosphamide or methotrexate. The best results were observed when the conjugate was administered for 24 h after subcutaneous inoculation of cancer cells, although the drug was shown to be effective also when administered to mice bearing tumours of a diameter less than 1 cm.

A similar strategy was used for experimental treatment of leukemias. To that purpose tumour-bearing mice were treated with genistein conjugated to B43 antibody which recognized the C19 antigen, present on the surface of B lymphocytes and absent from plasma cells. CD19 is an adaptor protein for Lyn tyrosine kinase amplifying signals transduction from nonreceptor Src tyrosine kinases. The authors expected that, after internalization, the genistein conjugate with anti-CD19 antibody would block efficiently the Lyn kinase activity, leading to apoptosis of leukemic cells. The study was performed on SCID mice bearing human acute lymphoblastic leukemia (ALL) or non-Hodgkin's lymphoma (Ek *et al.*, 1998). It was found that the conjugate was more effective therapeutically than cytostatics routinely used for treatment of this kind of leukemias. Subsequently, in order to determine whether the conjugate could be used for treatment of humans, a toxicity test was performed on cynomolgus monkeys (Messinger *et al.*, 1998). The animals obtained various intravenous doses (1.7 to 6.8 μmol/kg per day) using various time schedules, and no toxic symptoms were observed during long term observation. At the same time it has been found that $t_{1/2}$ for the conjugate in the circulation was within the 10–23 h range. These highly encouraging results inclined the authors to perform phase I clinical study.

In the clinical trial (Uckun *et al.*, 1999) genistein conjugate was applied to seven children and eight adults with acute lymphoblastic leukemia, for whom conventional therapies had failed. The conjugate was administered intravenously at daily doses of 0.1–0.32 mg/kg for 10 days or weekly at an appropriate dose. No severe side effects were observed and one remission and two temporary responses were noted, thus the authors assessed those results as encouraging.

Complexes of genistein with piperazine

The low solubility of genistein in aqueous solutions is regarded as a serious drawback limiting its therapeutic application. In order to increase the solubility, a complex of genistein with piperazine was obtained in which the OH group at C-7 of genistein was bound to piperazine nitrogen by a hydrogen bond (Fig. 4) (Mazurek *et al.*, 1998). The authors suspected that such a complex could function as a kind of prodrug slowly releasing genistein to aqueous media. Although the conjugate showed over 500 times better solubility than free genistein, the rate of genistein release was unsatisfactorily slow. Moreover, the *in vitro* cytotoxicity against HL-60 human mieloblastoma cells was similar for genistein and its conjugate with piperazine (Polkowski *et al.*, 2000). In any case, the persistence of the complex in diluted aqueous solutions can be challenged on thermodynamic grounds.

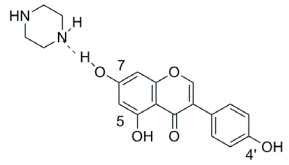


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Figure 4. Adduct of genistein and piperazine
According to Mazurek *at al.* (1998).

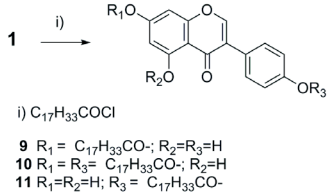
Lipophilisation of genistein

In order to improve the biological activity of genistein by increasing its rate of cellular uptake and to extend its stability as well as blood circulation Meng *et al.* (1999b) synthesized a number of fatty acid esters of genistein. Those were genistein 4'-stearate, genistein 7-stearate, genistein 4',7-distearate, genistein-4',-oleate, genistein-7-oleate and genistein 4',7-dioleate (Fig. 5). The authors expected that these highly lipophilic genistein derivatives would be effectively incorporated into low density lipoproteins (LDLs), and that, similarly to cholesterol, they would be effectively transported into various tissues and taken up by cells *via* receptor-mediated endocytosis.

When tested for use in artificial LDLs, all the genistein oleates were much more effectively incorporated into these liposomes as compared to free genistein or the genistein stearates. Subsequent studies of the antiproliferative effect of the LDLs loaded with genistein derivatives against U937 cells (human leukemia) revealed that genistein-7-oleate and genistein-4',7-dioleate effectively inhibited incorporation of radioactive thymidine into DNA, while genistein-4'-oleate was ineffective (Meng *et al.*, 1999a). The mechanism of the antiproliferative activity of the selected fatty acid esters of genistein has not been determined, however.

GENISTEIN DERIVATIVES AS NO DONORS

Nitrogen oxide (NO) is a well-known regulatory molecule involved in various cellular processes. Specifically, NO is involved in the regulation of contractility of vasculature thus maintaining vascular homeostasis (Miller & Megson, 2007). Having in mind that various vascular diseases can be caused by aberrant functioning of tyrosine kinases, Matsumoto *et al.* (2005) decided to synthesize genistein derivatives which should potentially be able to inhibit tyrosine kinase activity and increase NO level. They synthesized two novel genistein derivatives 7-[(4-nitroxy)butyroyl]-genistein (**12**) and 7-[(4nitrooxymethyl)-(α-methyl)phenylpropanyl]-genistein (**13**) (Fig. 6), and assessed their ability to relax rat endothelium-denuded aortic strips. Both derivatives and genistein itself induced aortic relaxation in the following



i) $C_{17}H_{33}COCl$

9 R_1 = $C_{17}H_{33}CO$-; R_2=R_3=H
10 R_1 = R_3= $C_{17}H_{33}CO$-; R_2=H
11 R_1=R_2=H; R_3 = $C_{17}H_{33}CO$-

Figure 5. Lipophilic esters of Genistein. According to Meng *et al.* (1999b)

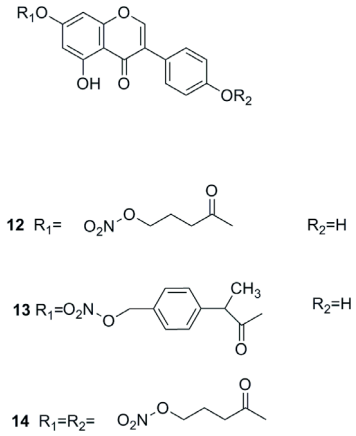


Figure 6. Genistein derivatives as NO donors
According to Matsumoto *et al.* (2005).

order: 7-[(4nitrooxymethyl)-(α-methyl)phenylpropanoyl] genistein > 7-[(4-nitroxy)butyroyl]-genistein > genistein. The relaxation induced by **12** and **13** was abolished by a guanylyl cyclase inhibitor, proving that the genistein derivatives indeed acted as NO donors.

When rat aortic strips in the experimental system described above were treated with lysophosphatidylcholine (LPC), a major phospholipid component of oxidized low-density lipoprotein (Ox-LDL), critically implicated in the atherogenic activity of Ox-LDL through an involvement of tyrosine kinase, the relaxation induced by **12** and **13** was abolished. The above observations confirmed the authors' expectations that the genistein derivatives **12** and **13** exhibit NO donor capacity and exert their inhibitory effect against tyrosine kinases.

Nitric oxide also plays an important role in bone metabolism. The drugs being NO donors effectively counteract bone mass loss occurring due to reduced rate of estrogen biosynthesis in postmenopausal women (Wimalawansa, 2000). What is particularly important, the NO donors not only slow down the rate of bone resorption, but also stimulate proliferation of osteoclasts (Hukkanen *et al.,* 2003).

In order to find a bifunctional derivative of genistein having both estrogenic properties suitable for hormone replacement therapy and being an effective nitric oxide donor able to restrain bone loss in postmenopausal women, Wang *et al.* (2007) synthesized genistein 7,4'-(nitroxy) butyrate (**14**). Its NO-releasing capacity was studied *in vitro* using MC3T3-E1 cells, an immature osteoblastic cell line derived from calvaria of newborn C57BL/6 mouse. It has been demonstrated that NO is released from derivative **14** less rapidly and for a longer time than from glyceryl trinitrate (GTN), the classical NO donor routinely used in medical treatment (Wang *et al.,* 2007). Using MTT assay and flow cytometry it was determined that **14** stimulated growth of MC3T3-E1 cells in a dose- and time-dependent manner, with the highest stimulatory effect observed for treatment of cells with 1 nM NO-genistein for 48 h, albeit the stimulation was weaker than that observed for an optimal concentration of estradiol. In order to determine whether NO-genistein is able to stimulate differentiation of osteoblasts the authors measured the activity of a bone-specific isoform of alkaline phosphatase and the expression of osteocalcin, a specific marker for late osteoblast differentiation, as well as the rate of formation of calcific deposition. All those assays showed that derivative **14** stimulated osteoclast differen-

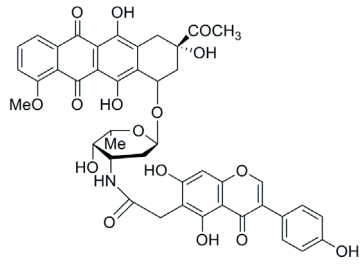


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Figure 7. Conjugate of daunomycin with genistein
According to Somjen (2003).

tiation and mineralization more effectively than genistein, glyceryl trinitrate or combination of the two. Although the effect of **14** was less pronounced than that of estradiol, Wang *et al.* (2007) concluded that **14** could be an interesting drug for treatment of postmenopausal osteoporosis.

GENISTEIN AS A VEHICLE OF SELECTED CYTOSTATIC DRUGS

It has been suggested that the affinity of genistein or its derivatives for estrogen receptors (ERs) could be used to target cytotoxic drugs to cancer cells that express membrane form of ER (Somjen *et al.*, 2003). The authors synthesized a conjugate of daunomycin and 6-carboxymethyl genistein (**2**), the derivative described above (Fig. 7). This conjugate (**15**) was used for treating NCI-H295R human adrenocortical cancer cells which express estrogen receptor on their surface. It was found that at low concentrations (0.3–30 nM) the conjugate inhibited cell proliferation more effectively than free daunomycin. However, at higher doses (300–3000 nM) the toxicities of free and genistein-conjugated daunomycin (**15**) were similar.

SYNTHETIC GENISTEIN DERIVATIVES OF ANTICANCER ACTIVITY

A significant step in the search for genistein derivatives with anticancer activity was the synthesis of nine genistein glycosides of which some turned out to inhibit proliferation of various cell lines *in vitro* (Polkowski *et al.*, 2004). The structure of the most active compound, (**16**) termed G21, is shown in Fig. 8.

This genistein derivative has the following structural characteristics: acetylated sugar hydroxyls, a double C=C bond in the sugar molecule binding directly to aglycone, α configuration of the genistein-sugar glycosidic bond, and localization of the sugar substituent at the C7-OH position in the genistein molecule. Further studies of the properties of G21 demonstrated that its IC_{50} was around 5 μM, thus approx. 10 times less than that of genistein. The structure of the molecule was stable, and its toxicity against cancer cells was higher than against normal ones (Popiołkiewicz *et al.*, 2005). A preliminary characterization of G21 concluded that this novel genistein glycoside did not hydrolyze under *in vitro* cell culture conditions and that the structural feature critical for its high cytotoxicity was the double C=C bond in the sugar moiety directly bound to C7 of the aglycone as well as acetylation of OH groups in the sugar residue (Ksycińska *et al.*, 2004).

A search for the mechanism of G21 cytotoxicity has revealed an unexpected feature of this compound. It was found that G21 is able to disrupt the microtubule network and to affect the structure of centrosomes, causing the appearance of aberrant mitotic spindles. In an *in vitro* assay the polymerization of purified bovine brain tubulin was inhibited by G21, indicating that this compound interacted with tubulin directly (Rusin *et al.*, unpublished). Moreover, treatment of cells with G21 led to the generation of micronuclei containing centromeric markers, which was not observed when cells were treated with genistein. Thus G21, unlike genistein, exhibits aneugenic properties. These observations reveal that, G21 is the first derivative of genistein able to substantially affect microtubule dynamics.

In our preliminary studies we also found that several genistein derivatives with a sugar moiety linked to genistein by a 2–5 carbon atom spacer possessed stronger antiproliferative activity. One of them blocked the cell cycle in the G2/M phase and destroyed the structure of mitotic spindle, thus resembled mode of action of G21 (Rusin *et al.*, unpublished).

The derivatives for which antiosteoporotic action had previously been reported (Wang *et al.*, 2005) were also screened for anti-proliferative potential (Li *et al.*, 2006). In their screening studies Li and coworkers tested 30 new derivatives (Fig. 3) (among them several ones not previously described) for their ability to inhibit proliferation of KB cells. They found several derivatives with increased antiproliferative potential.

In another study aimed at obtaining novel genistein derivatives with potential anticancer activity Zhang *et al.* (2007) synthesized a series of 24 derivatives containing the alkyl spacer between C7 of genistein and amines. One of the main goals was to determine how the length of the spacer linking amine residue to the genistein influences cytotoxicity of these novel genistein derivatives. The cytotoxicity study was performed using KB and K562 cells and it was found that only few genistein derivatives were more active than the parent drug, having IC_{50} of about 7–10 μM (Zhang *et al.*, 2007). The most active were the derivatives with the amine group sepa-

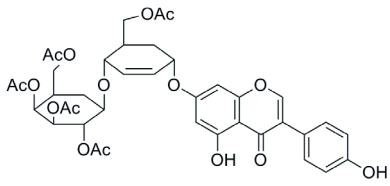

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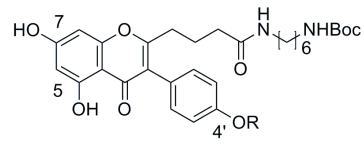
Figure 8. Glycoside of genistein inhibiting microtubule polymerization
According to Rusin *et al.* (2009).



17 R=H
18 R=Me

Figure 9. Amide of 2-carboxypropylgenistein exhibiting high antiproliferate activity
According to Kohen *et al.* (2007).

rated from genistein by a three-carbon chain. However, the mechanism of the increased cytotoxic activity has not been determined.

A novel genistein derivative exhibiting significantly higher antiproliferative activity than the parent drug was recently obtained by Kohen *et al.* (2007) by attaching an N-tert-butoxycarbonylo-1,6-diamino-hexane group to C2 of genistein (Fig. 9). Although this novel genistein derivative did not show estrogenic activity, it may interact somehow with estrogen receptor. The antiproliferative activity was shown to be different in estrogen-sensitive cancer cell lines expressing ERα and ERβ mRNA at different ratios. The highest antiproliferative effect measured by radioactive thymidine incorporation was observed for an estrogen-sensitive colon cancer cell line (320DM), and the lowest for an ovarian cancer cell line (A2780). Interestingly, the genistein derivative was more toxic for cells that preferentially expressed mRNA for ERβ relative to ERα. Moreover, the drug in general inhibited more effectively the proliferation of cancer cells than that of normal vascular smooth muscle cells.

Apart from the genistein derivative described above, Kohen *et al.* (2007) also synthesized similar derivatives of biochanin (2-[3-carboxy-(6-tert-butoxycarbonylamino)-hexylamino-propyl]-7,5-dihydroxy-4'-methoxyisoflavone) and daidzein (5-[2-[3-(4-hydroxy-phenyl)-4-oxo-4*H*-chromen-7-yloxy]-acetylamino]-pentyl)-carbamic acid tert-butyl ester). These compounds also did not exhibit estrogenic properties. Of all the derivatives of isoflavones tested by Kohen *et al.* (2007), the highest antiproliferative activity was shown for the N-t-Boc derivative of 7-(*O*)-carboxymethyl daidzein.

A series of chemically modified daidzein derivatives (2,7,4' substituted) were also prepared by Davis *et al.* (2008). Those compounds were found to bind with low affinity to ERβ receptor and to inhibit proliferation of hormone-dependent and hormone-independent breast cancer cell lines, thus confirming that isoflavone derivatives act on multiple signaling pathways, not necessarily mediated by ER, leading to the activation of cell death mechanisms.

GENISTEIN DERIVATIVES WITH ANTIMICROBIAL AND ANTIPARASITIC ACTIVITY

Various flavonoids and isoflavonoids are recognized as antibacterial, anti-viral and anti-fungal agents (Cowan, 1999). Genistein has with antimicrobial properties (Dixon & Ferreira 2002; Verdrengh *et al.*, 2004; Hong *et al.*, 2006; Ulanowska *et al.*, 2006). Cell survival studies suggest that genistein is a bacteriostatic rather than a bactericidal agent (Ulanowska *et al.*, 2007). Some reports indicate that the potent antibacterial properties of genistein shown *in vitro* may be mediated by the stabilization of the covalent topoisomerase II–DNA cleavage complex (Verdrengh *et al.*, 2004). Nevertheless, the exact mechanism of antimicrobial action of genistein remains largely unknown. The concentration of genistein necessary for antimicrobial action is relatively high (100 μM).

The derivatisation of genistein leading to an increased antibacterial and antifungal activity was reported by Zhang *et al.* (2008). They prepared three series of derivatives in which the genistein ring system was linked to the heterocyclic moieties with 2-carbon, 3-carbon or 4-carbon spacers (Fig. 10). Among the compounds tested some (**19**, **20**, **21**, **22** and **23**) exhibited good antibacterial activities, while **19** also showed notable antifungal activity. The activity of the mentioned derivatives was several fold better than that of genistein.

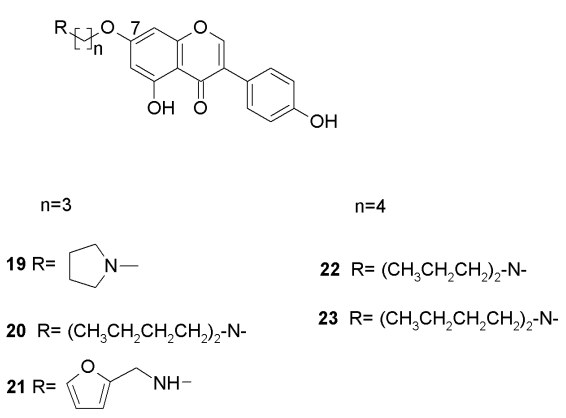
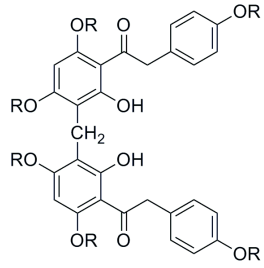


Figure 10. 7-O-modified derivatives of genistein exhibiting antibacterial activity
According to Zhang *et al.* (2008).

An antimicrobial activity of genistein derivatives was also described by Li *et al.* (2008). They synthesized and tested 14 new deoxybenzoin derivatives of genistein (Fig. 11) and found that dimeric deoxybenzoin (**24**–**28**) derivatives are generally more active than genistein and deoxybenzoins against selected microorganisms.

Genistein derivatives are also described as potential agents to treat parasitic diseases. Interesting examples suggesting that isoflavones have the potential for anti-protozoan therapies were reported by Gargala *et al.* (2005) and Stachulsky *et al.* (2006) and for anti-helminthic therapies by Naguleswaran *et al.* (2006). Gargala and coworkers examined activities of fifty-two dihydroxyisoflavone and trihydroxydeoxybenzoin derivatives on *Neospora caninum, Sarcocystis neurona* and *Cryptosporidium parvum* development *in vitro*. They also assessed the effects of two agents selected in *in vitro* screening: 3'-bromo and 4'-bromo genistein in *Cryptosporidium parvum*-infected immunosuppressed gerbils. They found more effective the abolishment of fecal microscopic oocyst shedding after administration of these two compounds than after nitazoxanide or paromomycin, two routinely used drugs.

Some of the derivatives described by Gargala *et al.* (2005) were also tested for inhibitory effects on the development of tapeworms *Echinococcus* sp. (Naguleswaran *et al.*, 2006). They found that two of the genistein deriva-



24 R= H;
25 R= Me,
26 R= $HOOCCH_2$-
27 R= $Br(CH_2)_3$-
28 R= $Br(CH_2)_4$-

Figure 11. Deoxybenzoin derivatives of genistein
According to Li *et al.* (2008).

tives (2'-bromo-and 6'-bromo genistein), induced considerable damage in *E. granulosus* protoscoleces, rendering them nonviable. Although genistein is a potent inhibitor of *Echinococus* larvae development, prolonged treatment with this isoflavone may cause adverse effects from the undesired stimulation of ER. The above mentioned genistein derivatives do not bind to ER and there is no risk of side effects caused by a phytoestrogen.

It is also worth mentioning that some of the isoflavone derivatives may act selectively, having excellent antiparasitic activity and lacking antibacterial activity (Stachulsky *et al.,* 2006). The absence of antibacterial properties is advantageous, because it allows avoiding the development of further resistant bacterial strains.

The examples mentioned above demonstrate that synthetic isoflavones exhibit distinct effects on parasites and could be potentially exploited further in the development of novel chemotherapeutic tools against infections by protozoa and larval-stage helminthes.

GENISTEIN AND ITS DERIVATIVES FOR TREATMENT OF MUCOPOLYSACCHARIDOSES AND CYSTIC FIBROSIS

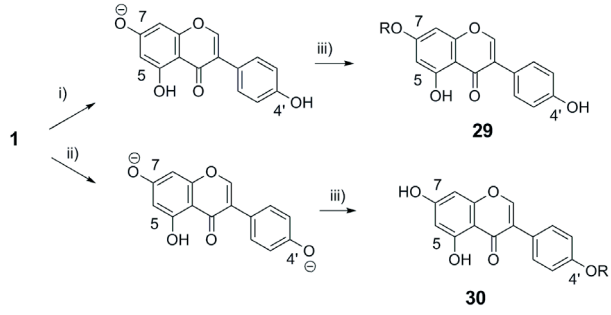
Mucopolysaccharidoses (MPs) are inherited metabolic disorders caused by mutations leading to dysfunction of one of the enzymes involved in the degradation of glycosaminoglycans (GAGs). An impairment of GAGs degradation leads to their accumulation in patients' cells and this causes dysfunction of tissues and organs. Genistein has been recently reported to inhibit the synthesis of GAGs in cultured fibroblasts from MPS patients (Piotrowska *et al.*, 2006). Prolonged cultivation of those cells in the presence of genistein resulted in the reduction of GAG accumulation and normalization of cells, as estimated from biochemical tests and electron microscopic analysis. Genistein also reduces lysosomal storage in peripheral tissues of mucopolysaccharide IIIB mice (Malinowska *et al.*, 2009). A recent pilot clinical study indicated that such a therapy may be effective in MPS III (Sanfilippo syndrome) (Piotrowska *et al.*, 2008). It must be noted, however, that genistein content differs in commercially available soy products. Some of them may be useless for MPS treatment and the use of pure synthetic genistein may be an option (Piotrowska *et al.*, 2009). The mechanism of genistein-mediated inhibition of GAG synthesis relies on epidermal growth factor (EGF)-dependent pathway (Jakóbkiewicz-Banecka *et al.*, 2009). Based on several human fibroblast culture experiments, it was found that some synthetic genistein derivatives inhibit GAG production by up to 90% in fibroblasts from MPS patients of various types (Kloska *et al.*, 2006).

Of 20 synthetic genistein derivatives tested with MPS III human fibroblast cultures, five showed an inhibitory effect on GAG synthesis similar to genistein or even higher.

Genistein was shown to partially activate the defective chloride channels (cystic fibrosis transmembrane regulator, CFTR) associated with cystic fibrosis (CF). Not only does it partially restore the CFTR activity but in addition it augments CFTR maturation and increases its localization at the cell surface (reviewed by Węgrzyn *et al.*, 2009). This dual mode of action along with its low toxicity places genistein among candidates for CF therapeutics. Pre-clinical studies with genistein have provided a basis for clinical trials with CF patients. Currently, a Phase II clinical trial is underway; it investigates the effects of combined treatment (4-phenylbutyrate and genistein) in CF patients with the ΔF508 mutation. (Clunes & Boucher, 2008). Although genistein derivatives for potential treatment of cystic fibrosis have not been studied extensively yet, the work of Gallieta *et al.* (2001) justifies the search for new compounds based on the structure of this isoflavonoid. Gallieta and coworkers generated a combinatorial compound library based on two lead compounds, flavones and benzo[*c*]quinoliziniums, which are believed to activate CFTR Cl2 conductance by direct interaction with the CFTR molecule. Several novel CFTR activators were identified. Interestingly, the structures of a high potency to activate CFTR, the 7,8-benzoflavones contained features of both flavones and benzo[*c*]-quinoliziniums.

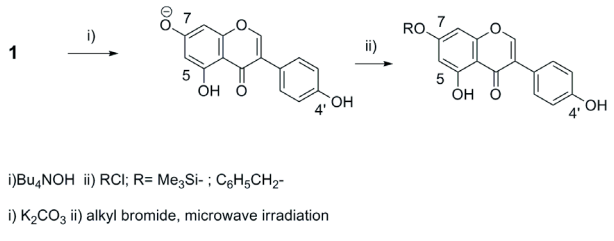
REACTIVITY OF GENISTEIN AND SYNTHESIS OF ITS ANALOGS

It is obvious from the structural formula of **1** that its multifunctional features offer ample opportunity for derivatization, but screening of the chemical literature allows one to conclude that this potential has not been explored extensively thus far. Most of the published studies have concentrated on functionalization of hydroxyl groups. By virtue of its three phenolic groups, at C-5, C-7 and C-4', genistein is a very weak Bronsted acid. In principle these groups can all be functionalized in typical O-acylation or O-alkylation reactions. The question of selectivity of such substitutions is related to the susceptibility for deprotonation, which has been studied spectroscopically; in conclusion, the acidity of phenolic groups in **1**, under a variety of aqueous environment conditions, is as follows: 7-OH > 4'-OH > 5-OH (Zielonka *et al.*, 2003). The corresponding rate constants differ by approximately two orders of magnitude between successive pairs of OH groups. Additionally, 5-OH is practically eliminated from competition because of its engagement in a strong intra-molecular hydrogen bond with the C-4 carbonyl group. Therefore, it has been postulated that selective substitution at 7-OH or 4'-OH can be achieved either directly, or stepwise, by adopting a protecting group strategy. Indeed, Lewis *et al.* (2000) in their studies have demonstrated that genistein monoanions, generated by the action of strong bases in organic solvents, can be selectively alkylated and acylated at C-7 (**29**), while the corresponding dianions obtained under similar conditions can be selectively substituted at C-4' when treated with one equivalent of a derivatizing reagent (**30**), pointing to the higher nucleophilicity of the less acidic phenolic group (Fig. 12). This approach has allowed ob-



i) strong base,1eq; ii) strong base, 3eq iii) RCl, 1eq ; R= palmitoyl, oleoyl,stearoyl

Figure 12. Selective acylation of genistein
According to Lewis *et al.* (2000).



i)Bu_4NOH ii) RCl; R= Me_3Si- ; $C_6H_5CH_2$-

i) K_2CO_3 ii) alkyl bromide, microwave irradiation

Figure 13. Regioselective alkylation of genistein
According to Wang *et al*. (2005).

taining a variety of lipophilic esters of **1**, by reacting its anions with palmitic, stearic and oleic acid chlorides.

However, the reactivity of **1** is not limited to simple ionic reactions depicted above. Phenolate anions are susceptible to secondary transformations initiated by Sequential Proton Loss Electron Transfer leading to the formation of hydroxyl radicals, which are particularly important for *in vivo* interactions of polyphenolic molecules (Musialik *et al*., 2009).

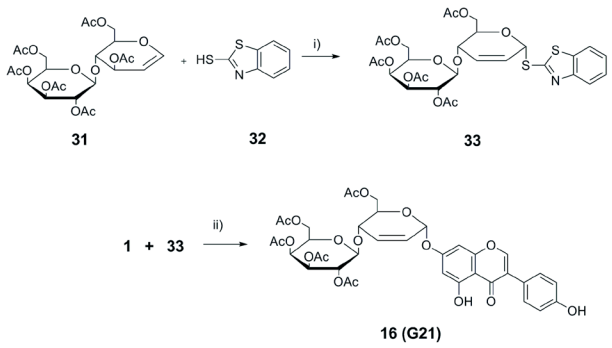
Attempts of direct functionalization of **1** have often been hampered by the difficulties in its solubilization under typical acylation/alkylation reaction conditions. In order to overcome this problem, sonication has been used extensively to activate reactants surface in heterogeneous reaction mixtures (Guilet *et al*., 1998; Li *et al*., 2002; Hofmann, 2003; Bonrath, 2004; Wang *et al*., 2006; Li *et al*., 2006; Zhang *et al*., 2008). In an analogous manner, microwave irradiation has been applied as a mean for enhancing molecular energy transfer to facilitate acylation and alkylation reactions involving **1** (Fig. 13) (Wang *et al*., 2005).

Although **1** is easily degraded under strong alkaline conditions, we found that it can form stable salts with strong bases. These salts can be useful intermediates for further derivatization of **1**. In particular, it has been demonstrated that one such intermediate, mono tetra-N-(n-butyl) ammonium salt of genistein greatly facilitates its selective silylation, acylation and alkylation (Grynkiewicz *et al*., 2004).

This finding allowed facile synthesis of a series of 7-O-benzylated derivatives of **1** that were later studied as molecular probes modifying experimental and biological lipid membranes (Środa *et al*., 2008).

Genistein easily undergoes single and double 7,4'-O-silylation in dimethylformamide and in the presence of imidazole as a base. Derivatives bearing tert-butyldimethylsilyl residues are isolable as stable intermediates, featuring some unexpected reactivity (Szeja *et al*., 2003). In particular, in the presence of a typical acylation (Ac_2O/Py) mixture the sililoxy group can be selectively replaced by an acetoxy residue. When phthalic anhydride is used as the acyl component, selective deprotection takes place.

Since genistein, like other flavonoids, occurs in nature principally as a glycosides, chemical glycosylation as a way of forming libraries of new chemically diversed compounds is an obvious strategy. Unfortunately, most methods known are not well suited for complex multifunctional aglycones (Grynkiewicz *et al*., 2008). The strategy of using lipophilic protecting groups, which facilitate solubility of substrates in organic solvents and reduce multiple reactive centers, proved, again, reasonably successful, even with traditional procedures like the Koenigs-Knorr method (Nishiyama *et al*., 1993; Al-Maharik & Botting, 2006). Glycosylation of unprotected



i) $InCl_3$, methylene chloride; ii) $Cu(OTf)_2$, methylene chloride

Figure 14. Stereoselective synthesis of genistein glycoside
According to Rusin *et al*. (2009).

genistein with acetobromoglucose, aimed at obtaining a derivative with 7-O-β-D-configuration was first described in 1943 by Zemplen and Farkas (1943) who reported 17% yield.

In recent years, owing to special deprotonation design, various procedures involving phase-transfer catalysis succeeded in attaining 7-O- as well as 4'-O- regioselectivity, also with relatively low yields (Lewis *et al*., 1998; Lewis & Wähälä, 1998). Another method of glycosylation of **1** involves exchange of an anomeric ester group. Thus when **1** was reacted with tetra-*O*-acetyl-α,β-D-ribofuranose in the presence of tin tetrachloride, this yielded an anomeric mixture of 4'-*O*-ribofuranosides (about 26%) in which the β-anomer prevailed (Boryski & Grynkiewicz, 2001).

With respect to the mechanism of intermediate carboxonium ion generation, the application of glycals to the synthesis of glycosides is similar to the ribofuranose case presented above. The obtained products, however, are unsaturated and rearranged with respect to the position of the leaving group (Ferrier rearrangement) (Ferier & Zubkov, 2003). Rather unexpectedly, this useful transformation failed to work in our hands when applied to **1**, although different glycals and a number of various Lewis acid catalysts were tried. Thus, unsaturated pyranosides of **1** were obtained by a stepwise procedure in which the rearrangement performed with water as the nucleophile was followed by esterification and anomeric exchange of the unsaturated ester, catalyzed by a palladium complex (Polkowski *et al*., 2004).

This reaction, when compared with model Ferrier rearrangement, suffers unfortunately from low regio- and stereoselectivity. Since one of the genistein unsaturated glycosides, G21, interfered with tubulin organization and cell cycle progression, resulting in pronounced cytostatic effect, a more selective method of its preparation was sought (Rusin *et al*., 2009). Consequently, for efficient preparation of G21, a 2-mercaptobenzotiazole intermediate was proposed as the leaving group, instead of a carbonate ester (Fig. 14).

Information on skeletal modifications of **1**, involving C–C bond formation is scant and not encouraging. A notable exception results from applying higher carboxylic acid esters to the isoflavone synthesis pathway, which involves cyclization of a deoxybenzoin type intermediate, giving rise to 2-substituted genistein derivatives which bear α-ketoalkyl substituents. Introduction of a phenolic (or thiophenolic) substituent at C-2 of the benzochromanone ring was achieved by exchanging methylsulfone substituent (Kim *et al*., 2003). Substitution at position 2

of the isoflavone skeleton is relatively easy to achieve, *via* a novel cyclization reaction in which 2-acyloxy deoxybenzoins can be transformed into 2-alkyl isoflavones (Pelter *et al.*, 1998; 1999).

Successful triple hydroxymethylation of daidzein (structural analog of **1** without the C-5 hydroxyl group) with aqueous basic solution of formaldehyde indicates that similar condensations might be also possible for **1** (He *et al.*, 2008). 6-Carboxymethyl genistein has been recognized as a novel synthetic estrogen receptor modulator and suitable substrate for preparing conjugates with anthracyclines (Somjen *et al.*, 2002). This compound was obtained in low yield by treatment of **1** with bromoacetic acid and sodium in propanol under heating (Kohen *et al.*, 1999; 2007).

Other structural modifications of genistein, like halogenation, nitration, sulfonation etc., are possible owing to the reactivity typical for aromatic compounds (Soidinsalo & Wähälä, 2004; Wang *et al.*, 2005).

The closest analogs of **1** are the compounds obtained by exchanging one or several atoms with their stable or radioactive isotope (typically deuterium or carbon 13 or 14). Such compounds are invaluable in studies of biodistribution, pharmacokinetics and metabolism because they are good markers in HPLC analysis with mass spectrometry detection (Prasain *et al.*, 2004; Grynkiewicz *et al.*, 2005). A number of special synthetic procedures have been designed for the synthesis of labeled genistein; they have been sucessfuly applied in an increasing number of biochemical and pharmacological studies (Wähälä & Rasku, 1997; Whalley *et al.*, 2000; Coldham & Sauer, 2000; Oldfield *et al.*, 2007).

CONCLUDING REMARKS

There is no doubt that genistein is, like so many other secondary metabolites, a semiotic molecule (Barbieri, 2008; Iriti & Faoro, 2009). The belief that compound **1** can be successfully modified to become a more selective and more efficacious biologically active agent has rational foundations but so far chemical synthetic efforts have been poorly synchronized with biochemical findings. Genistein has become an obvious candidate for drug development because of its known molecular targets, but also out of the pressing needs for improvement of bioavailability and slowing down its *in vivo* conjugation and excretion. Interestingly, despite the known shortcomings of this secondary metabolite, there are numerous genistein-containing dietary supplements; this is accompanied by a remarkable persistence in the efforts to elevate the substance to the level of a registered active pharmaceutical ingredient (Grynkiewicz, 2002). Presently over 20 clinical trials of compound **1** are carried out in the USA alone, mainly for hormone-dependent ailments, including menopause symptoms, osteoporosis, and cancer (including its chemoprevention). At least three pharmaceutical products containing a defined dose of compound **1** made it to the market with clear medical indications: GCP™ (genistein-containing polysaccharide, Amino Up Chemical Co., Japan) as an anticancer drug; and Bonistein™ (DSM Nutritional Products, Netherlands) and Fosteum™ (Primus Pharmaceuticals, USA), both for improving osteoporotic bone structure (Yuan *et al.*, 2003; Ullmann *et al.*, 2005; Squadrito *et al.*, 2009).

This review testifies to the considerable synthetic effort made towards "better genistein" or "more efficient pro-genistein". Biological activity of the novel synthesized genistein derivatives seems to indicate ample space for designing new drugs; increasing structural complexity of the parent compound can bring about incremental increases of efficacy, radical changes in selectivity or even the appearance of a new mechanism of action. Although some new leads have emerged, the way to a validated drug candidate still seems quite distant.

It should not be overlooked that, besides adding new functionalities to the parent structure by chemical derivatization, an opposite tendency has also been followed, i.e. reducing some functional groups from compound **1.** This was apparently inspired by findings concerning isoflavone metabolism in experimental animals and humans (Setchell *et al.*, 2002). (*S*)-Equol, identified as the main mammalian metabolite of daidzeine (minor isoflavone constituent of legumes), has indeed been shown to exert a much more powerful estrogenic action than plant isoflavones. Equol can be relatively easily obtained synthetically, as well as by biotransformation, which makes it an interesting candidate for drug development (Heemstra *et al.*, 2006). The reductionist approach to compound **1** structure–activity relationship resulted in two more synthetic compounds emerging in new drug registration area. First, phenoxodiol (dehydroequol) is a new FDA fast-track-to-registration drug investigated as a chemosensitizer for platinum and taxanes (Silasi *et al.*, 2009). The second drug, ipriflavone, which was designed not to affect estrogenic receptors and is thus devoid of free hydroxyl groups, first became a hit as an over-the-counter non-steroidal anabolic, but was also registered as inhibitor of bone resorption and anti-osteoporosis drug (Agnusdei & Bufalino, 1997).

It is especially interesting that in the recent studies particular attention is paid to genistein derivatives as effective radio- and chemosensitizers. As stated above a substantially greater effort is required in investigating more thoroughly the cytotoxicity mechanisms of novel genistein derivatives. The chemistry of compound **1** remains opportunistic and limited to easy-to-perform procedures. Undoubtedly, more information about specific biological targets of genistein derivatives obtained so far is needed before a good drug candidate can emerge from the new compound libraries presented in this review.

Acknowledgements

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