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# BIOLOGICAL PROPERTIES OF GENISTEIN. A REVIEW OF *IN VITRO* AND *IN VIVO* DATA

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**Abstract:** Genistein – a soy derived isoflavone has recently attracted much attention of the medical scientific community. This compound was found to be a potent agent in both prophylaxis and treatment of cancer as well as other chronic diseases. The great interest that has focused on genistein led to the identification of numerous intracellular targets of its action in the live cell. At the molecular level, genistein inhibits the activity of ATP utilizing enzymes such as: tyrosine–specific protein kinases, topoisomerase II and enzymes involved in phosphatidylinositol turnover. Moreover, genistein can act *via* an estrogen receptor–mediated mechanism. At the level one step higher, i.e., at the cellular level, genistein induces apoptosis and differentiation in cancer cells, inhibits cell proliferation, modulates cell cycling, exerts antioxidant effects, inhibits angiogenesis, and suppresses osteoclast and lymphocyte functions. These activities make genistein a promising innovative agent in the treatment of cancer. Additionally, genistein health beneficial effects have been shown in osteoporosis, cardiovascular diseases and menopause. Genistein was also successfully used as an immunosuppressive agent both *in vitro* and *in vivo*. All these effects at the three biological levels of action need varied genistein concentrations and only some of them are relevant in people consuming soy–rich diet. The others would occur after purified genistein administration at higher doses. The main genistein advantage as a potential drug is its multidirectional action in the live cell and its very low toxicity.

**Keywords:** genistein, (iso)flavones, protein tyrosine kinase, topoisomerase II, apoptosis, differentiation, angiogenesis, cancer, osteoporosis, cardiovascular disease, immunosuppression, toxicity.

**The abbreviations used are**: **ABC** – ATP Binding Cassette; **CFTR** – Cystic Fibrosis Transmembrane Conductance Regulator; **DAG** – Diacylglycerol; **EGF** – Epidermal Growth Factor; **ER** – Estrogen Receptor; **HDL** – High Density Lipoprotein; **IGF** – Insulin Like Growth Factor; **IP3** – Inositol–1,4,5–triphosphate; **LDL** – Low Density Lipoprotein; **LPS** – Lipopolysaccharide; **MDR** – Multidrug Resistance; **MRP** – Multidrug Resistance–associated Protein; **NGF** – Nerve Growth Factor; **PDGF** – Platelet Derived Growth Factor; **PGE2** – Prostaglandin E2; **Pgp** – P–glycoprotein; **PIP** – Phosphatidylinositol–4–phosphate; **PIP2** – Phosphatidylinositol–4,5–bisphosphate; **PLC** – Phospholipase C; **PTH** – Parathyroid Hormone; **PTK** – Protein Tyrosine Kinase; **ROS** – Reactive Oxygen Species; **RS6K** – Ribosomal S6 Kinase; **TGFB** – Transforming Growth Factor Beta; **topo II** – Topoisomerase II.

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## 1. Genistein from discovery to applications

Genistein is a natural compound belonging to the class of isoflavones. While isoflavones are widely distributed in the plant kingdom, the concentration of these compounds is relatively high in legumes and in particular in soybean. The concentration of the predominant soy isoflavone, genistein, in the majority of soy food materials ranges from 0.2–1 mg/g, mostly as various forms of glycosidic conjugates (1,2). Genistein is present in Trifolium species too, however, it was also isolated from fermentation broth of various kinds of microorganisms (*Streptomyces sp., Pseudomonas sp.*) (3,4). Genistein was not formed by the microorganisms as such, but rather was derived from hydrolysis of the glycosidic conjugates present in the soymeal used as a protein source. The interest in the mechanism of genistein action originated in the discovery that the compound, like other isoflavones, possesses a remarkable estrogenic activity (so they are termed phytoestrogens, i.e., plant–derived estrogens). As early as 1931, Waltz (5) ascertained genistein to be the major phytoestrogen in soybean. In 1946 isoflavones, including genistein, were found to be the cause of infertility effects in sheep grazing on subterranean clover in Western Australia (6). Prolonged grazing resulted in permanent infertility after three seasons, the syndrome is known as the clover disease. Forty years later a similar problem with reproductive failure and additionally venocclusive liver disease occurred in captive cheetahs fed on a diet containing soybean. In this case, it was found that soy–derived genistein, acting as a weak estrogen, was the main reason for the animal health problems (7). The next important finding concerning genistein was in 1981 when Asahi et al. isolated from a culture filtrate of a streptomycete a new substance which induced differentiation of mouse leukemia cells and was not toxic in mice. They called it differenol A but subsequently it turned out to be identical with genistein. However, the real stimulus triggering the interest in genistein in medical scientific community was by the finding in 1987 that this compound is a specific and potent inhibitor of protein tyrosine kinases, not affecting serine/threonine kinases (8). The number of published reports on genistein increased from a sleepy one to six papers a year prior to 1987 to over 200 a year after 1993 (9). Since it was recognized that many oncogenes (cancer causing genes) code for tyrosine kinases, therefore, the discovery of a substance that can block this class of enzymes caused a hope for a progress in the cancer treatment. Moreover, successive epidemiological studies strongly suggested that a diet rich in soybean and hence genistein, has beneficial effects on human health, especially in terms of cancer prevention. These findings further inspired many researchers all over the world to elucidate the mechanism of genistein action at the cellular and molecular level. In 1989, Markovits et al. reported genistein to be an inhibitor of topoisomerase II, a nuclear enzyme involved in cellular replication (10). Four years later, the isoflavone was found to be a modulator of drug accumulation in non–Pgp MDR cells (non P–glycoprotein mediated multidrug resistance), unaffecting Pgp MDR cells (11). Subsequently, Zhou and Lee (1998) discovered that genistein can blunt the response of cancer cells to stress by inhibiting stress genes expression. The value of genistein as a potential anticancer drug was also appreciated by National Cancer Institute (NCI). In 1995, the Division of Cancer Prevention and Control, NCI recommended genistein for clinical development as a cancer chemopreventive agent (12). Despite the fact that genistein has not been registered as drug, numerous companies offer it for sale as a standardized isoflavone extract from soy. It is advertised as health beneficial supplement of a daily diet.

## 2. Biological properties of genistein

Despite the relatively simple chemical structure of genistein its mode of action remains still fairly unexplored. Numerous experiments that have been undertaken show that genistein interferes with many biochemical pathways and its mode of action in the live cell is complex and multidirectional. Thus, there are defined several biochemical targets of genistein in the cell and they cover:

a) Protein tyrosine kinases,
b) Topoisomerase II,
c) Enzymes involved in phosphatidylinositol turnover,
d) Estrogen receptors,
e) ABC transporters,
f) Other proteins such as: Ribosomal S6 kinase; nuclear factor Y/CCAAT; TGFB; CFTR; aromatase; type I 17B–hydroxysteroid oxidoreductase.

The first four proteins are the main targets and their interactions with genistein are well documented. However, each of the above listed proteins may partly serve to account for varied genistein effects that can be observed in cell or tissue cultures. At

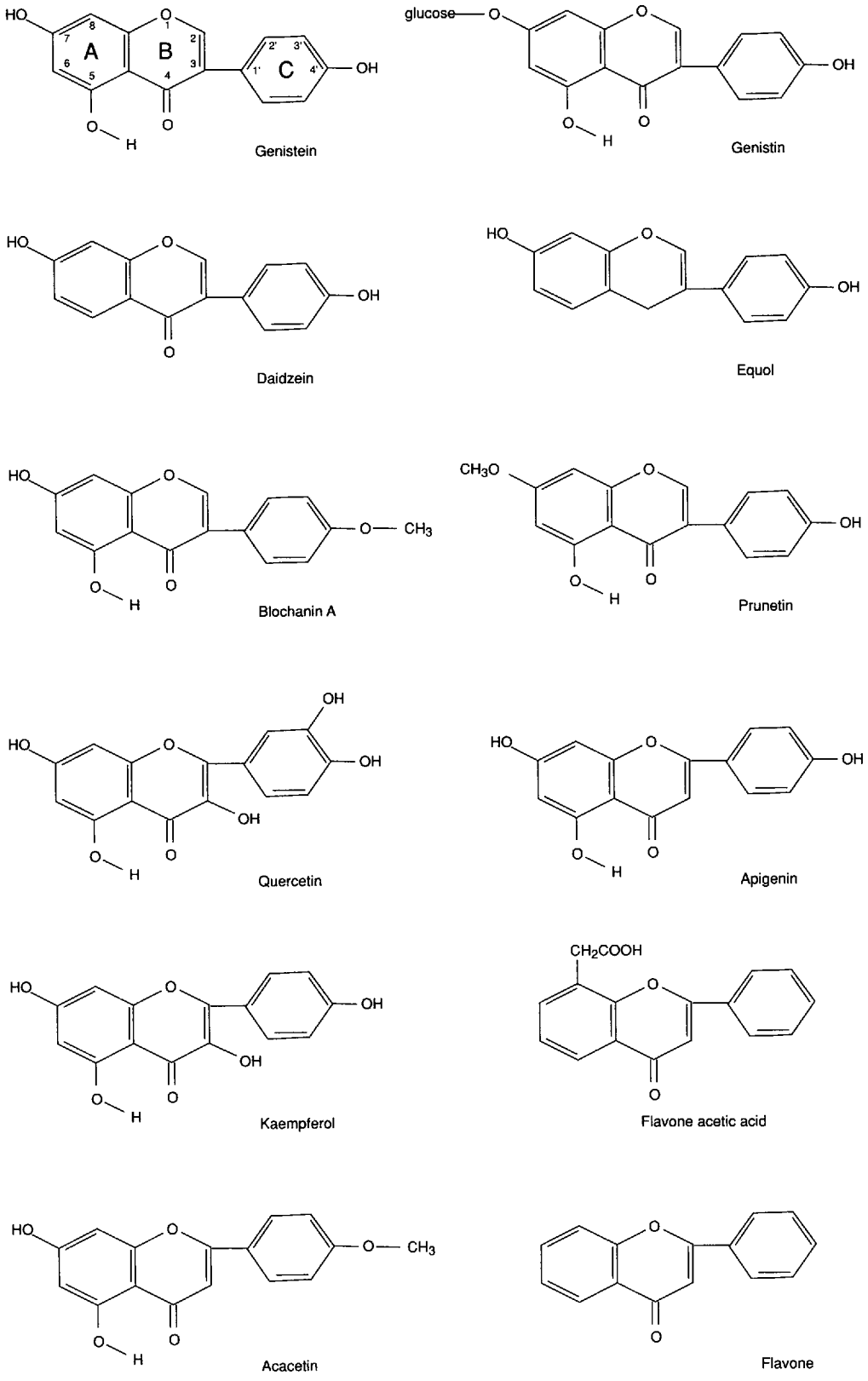


Figure 1. Chemical structures of (iso) flavonoids.

this „one step higher" level of action there are the following biological activities of genistein, explored mainly in cancer cells (a–f) but not only (g–j). Most of them have also been corroborated in experimental animal models.

a) Induction of apoptosis,
b) Induction of differentiation,
c) Inhibition of cell proliferation,
d) Modulation of cell cycle progression,
e) Antioxidant effects,
f) Reversal of multidrug resistance to anticancer drugs,
g) Inhibition of angiogenesis,
h) Suppression of osteoclastic function,
i) Suppression of lymphocyte activation and proliferation,
j) Mast cell stabilization and mild anti–inflammatory properties.

In view of all these genistein effects, it is not surprising that there are at least a few disorders for which genistein could be possibly used. Thus, the recent studies are focused on application of this natural isoflavone in the prophylaxis as well as treatment of the following diseases:

a) Cancers,
b) Postmenopausal bone lose and osteoporosis,
c) Cardiovascular diseases,
d) Menopause,
e) Other diseases such as familial chronic nose bleed disorder; cystic fibrosis; allergies.

The biological properties of genistein described above are divided into three parts. The first one concerns molecular targets of the compound in the live cell. The next one refers to genistein–mediated effects observed in cell or tissue cultures and is extended to include animal studies. The third part of the paper is devoted to the potential usage of genistein in the treatment of certain diseases. Such precise separation of respective biological properties of genistein enables a thorough study to be undertaken on the pathways leading from receptor–ligand (i.e. target protein–genistein) interactions through the impact of such interactions on the whole cell to end with the therapeutic effects observed in animal/human body.

## 3. Mechanisms of genistein action at the molecular level

Genistein has been shown to be an inhibitor of several intracellular enzymes. Most of them share a common property, namely, they utilize ATP in their enzymatic turnover (protein tyrosine kinases, topoisomerase II, phosphatidylinositol kinases, ABC transporters). The mechanism for the suppression of such diverse group of enzymes, hardly related to each other, is probably by genistein binding with a common, highly conservative sequence at, or near to, the ATP–binding domain. That results in competitive inhibition with respect to ATP, although there is no structural relationship between ATP and genistein (8). This mode of action accounts for the majority of genistein activities demonstrated in various experimental models. However, there is also a next reason why the isoflavone can modulate diverse biochemical processes in the cell. That is its structural and functional resemblance with estrogens, female steroid hormones which are well known to play numerous important roles in the cellular life.

### 3.1. Genistein as a protein tyrosine kinases inhibitor

Protein tyrosine kinases (PTKs) are critical components of the biological control networks that govern cellular growth and differentiation. These broad class of enzymes include receptor tyrosine kinases which play a crucial role in the signal transduction from the outer cellular environment into the interior of the cell. Receptor tyrosine kinases are activated by various peptide growth factors such as EGF (epidermal growth factor), IGF (insulin like growth factor), PDGF (platelet derived growth factor), NGF (nerve growth factor) etc. Extracellular ligand binding is the first step that triggers changes in the receptor protein (dimerisation, autophosphorylation) which then acquire intrinsic protein tyrosine kinase activity. Subsequently such receptor catalyzes phosphorylation of tyrosine residues of target proteins what finally leads to a complex array of cytoplasmic and nuclear events such as other protein phosphorylation, enzyme activation, second–messenger generation and transcription of immediate early genes in the nucleus (13). Apart from receptor, there are also cytoplasmic and nuclear protein tyrosine kinases and they are involved in regulation of various biochemical processes in the cellular life, too. Some PTKs actively participate in regulating the cell cycle progression e.g., pp34cdc2 which is the crucial enzyme controlling entrance in mitosis. Therefore, no wonder that PTKs have been shown to play an important role in the lymphocyte and osteoclast activation as well as in the stimulation of other specialized mammalian cells e.g., mast cells (14), eosinophils (15), macrophages (16), platelets (17), neutrophils (18). In healthy cells, PTKs activity is tightly regulated and a finely tuned balance between tyrosine kinases and phosphatases is essential for normal cell function. On the contrary, per-

manent increased level of tyrosine phosphorylation is implicated in many cancers since about one–half of the known oncogenes encode either membrane–bound receptors with tyrosine kinase activity or intracellular proteins undergoing or catalyzing tyrosine phosphorylation. These mutated enzymes phosphorylate target proteins in the signal transduction cascade what leads to malignant transformation and uncontrolled tumor growth. For example, such oncogenic PTK is the HER2 kinase found in mammary and ovary tumors in women and associated with unfavourable prognosis. Moreover, retroviral protein tyrosine kinases e.g., v–src, v–abl, v–erbB are also responsible for changing normal cells into cancerous cells *via* promoting proliferation, protection against apoptosis and blocking of cellular differentiation. Accordingly, it is a hopeful way to overcome cancer by using an inhibitor of PTKs which might be a very potent antitumor agent as well as a useful tool for understanding physiological and pathological role of tyrosine phosphorylation.

In 1987, genistein was proved to be a specific and potent inhibitor of PTK activity, with little inhibitory effect on serine/threonine kinases. *In vitro* in cell–free systems genistein inhibited the activity of several, both oncogenic and receptor, tyrosine kinases. This isoflavone turned out to be a very potent inhibitor of EGF–receptor activity, as measured by autophosphorylation ability. In this case, $IC_{50}$ value was only 2.6 μM/l (8). The molecular mechanism of genistein interaction with tyrosine–specific protein kinases has been investigated for EGF receptor as a kinase and histone H2B as phosphate acceptor. Kinetic analysis revealed that genistein is a noncompetitive inhibitor with respect to histone and leads to the formation of nonproductive enzyme–substrate complexes. By contrast, the inhibition was competitive with ATP as a second substrate, a phosphate donor. Since genistein bears no structural relationship with ATP, the true competition for exactly the same site as that utilized by ATP is rather excluded. Most likely, genistein binds to the highly conservative sequence at, or near to, the catalytic domain of the enzyme. Such sequence might be the common target for genistein in all PTKs. The hypothesis may be confirmed by the fact that primary amino acid sequence of catalytic subunit of PTKs is closely related to each other. This may also serve to explain the specificity of genistein for PTKs and the lack of activity towards serine/threonine kinases which are weak homologues with PTKs. Indeed, serine/threonine kinases such as protein kinase C (PKC) and cAMP–dependent protein kinase were scarcely inhibited by genistein concentrations as high as 370 μM/l (8). Nevertheless, genistein does not inhibit all PTKs equally, and some of them e.g., p94 (cytosolic PTK of HeLa cells) can be not inhibited at all, suggesting specificity of genistein towards respective PTK (19). Some of the protein tyrosine kinases inhibited by genistein in cell–free systems are listed in Table 1 (8).

Table 1. Exemplary protein tyrosine kinases inhibited by genistein.

| PTK | PTK substrate | $IC_{50}$ μM/l |
|---|---|---|
| EGF–R | EGF–R (autophosphorylation) | 2.6 |
| EGF–R | Histone H2B | 22.2 |
| Oncogene products: | | |
| v–src | IgG | 29.6 |
| v–src | Casein | 25.9 |
| gag–fes | gag–fes (autophosphorylation) | 24.1 |

*In vitro*, in intact cells, genistein–induced PTK inhibition was also reported but under these conditions substantially higher genistein concentrations had to be employed than in cell–free systems. For example, Akiyama et al. have examined the phosphoamino acid content of proteins of A431 cells treated with 150 μM/l genistein. They ascertained significant decrease in phosphotyrosine level of EGF–receptor and other cellular proteins. So the investigators concluded that genistein exerts PTK inhibitory effects in whole cells (8). Similar conclusions have been drawn by Spinozzi et al. Basing on their own studies with Jurkat T–leukemia cells, they have revealed that genistein treatment (111 μM/l) resulted in substantial diminution of tyrosine–phosphorylated proteins (20). In another study with MCF–7 breast cancer cell line, 100 μM/l genistein abolished proliferative response to insulin stimulation accompanied by intracellular tyrosine phosphorylation (21). These effects obtained for MCF–7 cells were likely to depend on impairment in the signal transduction cascade from tyrosine kinase receptors, although direct biochemical evidence had not been provided. Genistein has also been shown to inhibit proliferation of various human prostate cancer cell lines, cultured *in vitro* and stimulated with EGF. Genistein's growth inhibitory effect paralleled that observed with tyrphostin, a synthetic PTK inhibitor, what was consistent with genistein being a PTK inhibitor (9).

However, in spite of aforementioned reports recent investigations indicate that in cancer cells genistein–induced growth inhibition may not be solely due to decrease in PTK activity even in those cells with constitutively increased tyrosine phosphorylation pattern. In experiments designed to determine whether genistein alters the tyrosine phosphorylation of EGF receptor in the prostate cancer cells following stimulation with EGF, genistein had no inhibitory effects even up to 185 μM/l. In contrast tyrphostin at its $IC_{50}$ for cell growth reduced EGF receptor tyrosine phosphorylation to control levels (9). Similarly, it was found that although genistein inhibits the EGF–stimulated growth of human breast cancer cell lines and normal human mammary epithelial cells it does not inhibit EGF receptor tyrosine autophosphorylation. Genistein also failed to inhibit insulin receptor autophosphorylation in rat adipocytes at 370 μM/l. Furthermore, insulin receptor, PDGF–receptor and NGF–receptor are competent to transmit mitogenic signals in whole cells despite genistein concentration over 111 μM/l (13). Therefore, based on these and other studies, genistein capacity to inhibit receptor–associated PTKs in intact cells has been called into question (9).

In short, although genistein is a specific and potent inhibitor of purified PTKs *in vitro* in whole cells this inhibition may be observed but at relatively high genistein concentrations or even may not be observed at all. These discrepancies may be due to such reasons as limited genistein penetration through cellular membrane and various sensitivity of tested cells to genistein. The latter effect could be caused by cellular metabolism, sequestration by binding proteins or competing with high levels of ATP (13). Consequently genistein may be not capable of reaching sufficient intracellular concentrations to block tyrosine residue phosphorylation to an extent detectable by an analytical method. However, despite these controversies as to genistein's effects on PTKs in whole cells, it is widely used as a reagent for the study of tyrosine–specific protein kinases. It is also extensively investigated as a potential drug especially in cancers where oncogenic PTKs are involved.

### 3.2. Genistein as a topoisomerase II inhibitor

Topoisomerase II (topo II) is a nuclear enzyme that actively participates in DNA replication, transcription and probably in DNA repair processes. Topo II is responsible for regulating DNA topology, chromatin condensation/decondensation, chromosome separation and maintaining proper DNA structure in general. Increased activity of this enzyme has been reported in rapidly proliferating cells, especially in S and $G_2$ phase of the cell cycle. In its catalytic cycle, topo II covalently binds with DNA then induces transient DNA strand breaks, enables other strand to pass through the breakage and finally rejoins cleaved ends of DNA. Some of the most potent antitumor agents currently used in cancer chemotherapy, inhibit topo II e.g., anthracyclines (adriamycin, daunorubicin), epipodophyllotoxins (etoposide, teniposide). These drugs bind to the enzyme and interfere the breakage–rejoining step of the DNA strand–passing reaction by formation of a stable drug–topo II–DNA ternary complex. It is called the cleavable complex since its denaturation leads to the appearance of DNA strand breaks (22). Various cell lines treated *in vitro* with subcytotoxic concentration of topo II–targeting drugs can become resistant following qualitative/quantitative changes of the enzyme. When one of such resistant cell lines was treated with genistein as an innovative anticancer agent, the cross–resistance was observed. It strongly suggested that topo II may be involved in the action of genistein, although the compound was thought to act through a PTK mechanism. In fact, further detailed study confirmed this hypothesis. Experiments performed *in vitro* with purified topo II have revealed genistein to inhibit the catalytic activity of the enzyme. The inhibition was assayed by following topo II–mediated decatenation of mitochondrial kinetoplast DNA to minicircles and small catenanes. In the presence of genistein, the decatenation activity of the enzyme gradually decreased starting at 20 μM/l with complete inhibition at high concentrations of genistein up to 370 μM/l (10). To gain more insight into the genistein inhibitory mechanism the compound was tested to stabilize the cleavable complexes. Supercoiled plasmid pBR322 DNA was used as a substrate of purified topo II. In such experimental model, the isoflavone stimulated the generation of cleavable complexes as evidenced by the formation of DNA strand breaks. At the same time genistein, unlike other topo II–targeting drugs, did not intercalate into DNA (10). Those *in vitro* results from cell–free experiments, indicating genistein to be a topo II inhibitor, were also reproduced in whole cells. For example, Chinese hamster lung cells treated with genistein (≥20 μM/l) accumulated protein–linked DNA strand breaks as shown by DNA filter elution (10). In another study employing two leukemia cell lines, genistein has also been reported to induce DNA strand breakage due to the inhibition of topo II (23). This DNA damage has been proposed to be a contributing factor in the effects of genistein on

cultured cells such as apoptosis, growth inhibition or differentiation, for instance.

### 3.3. Genistein as an inhibitor of phosphatidylinositol turnover

Inositol–1,4,5–triphosphate (IP3) is an important molecule involved in cellular pathways of signal transduction. IP3 level rapidly increases in response to phospholipase C (PLC) activation. PLC hydrolyses membrane bound phosphatidylinositol–4,5–bisphosphate (PIP2) to produce IP3 and diacylglycerol (DAG). IP3 and DAG are the signaling molecules so called second messengers. The former releases $Ca^{2+}$ from intracellular compartments while the latter activates protein kinase C. Then, both signaling molecules undergo degradation. PIP2 is restored in a series of phosphorylation catalyzed by PI kinase (1–phosphatidylinositol 4–kinase) and PIP kinase (1–phosphatidylinositol–4–phosphate 5–kinase). The steady–state signal transduction activity as measured by PLC, PI and PIP kinase activities and IP3 concentration is markedly up–regulated in various types of cancerous cells (24). Additionally, these activities may also be elevated in normal, healthy cells following their timulation to perform physiological (or pathological) functions. Genistein is able to block both PI and PIP kinase and hence reduce IP3 concentration, what finally modulates signal transduction program (24,25). Consequently, biological effects of genistein on various types of cells are sometimes ascribed to the inhibition of phosphatidylinositol turnover (26). Some evidence supporting this concept came from a study on three myelomonocytic leukemia cell lines HL–60, ML1, U937. In this experiment, genistein ability to induce cancer cells differentiation was compared to methyl 2,5–dihydroxycinamate as a selective PTK inhibitor and to psi–tectorigenin which blocks only phosphatidylinositol turnover. These investigations pointed to the inhibition of inositol phospholipid metabolism as the main mechanism to explain genistein mode of action in those cells. Similar conclusion was drawn from studies performed with thrombin stimulated human platelets: In this case genistein did not affect protein tyrosine phosphorylation, serotonin release or PLC activity while cellular $Ca^{2+}$ level was substantially decreased. In order to clarify the mechanism by which genistein counteracts $Ca^{2+}$ mobilization, its effect was examined on platelet phosphatidylinositol turnover. From the results obtained, it has been concluded that genistein inhibited PIP kinase activity, what was reflected in diminished production of PIP2 and IP3 in the platelets (25).

### 3.4. Genistein and estrogen receptors

The structure of genistein to some extent resembles that of estrogenic steroids, so it is not surprising that it exhibits a quite significant estrogen–like activity. The question if this isoflavone acts as a pure estrogen or whether as a mixed estrogen agonist–antagonist still remains to be answered because some contradictions exist in papers published in this area. However, the majority of reports support the former view, pointing to the estrogenic activity of genistein. Thus, genistein has been found to bind to estrogen receptor (ER) with a relative affinity about 100–1000 – fold lower than that of estradiol. Consequently, this isoflavone competes with estradiol to displace it from its binding sites. Moreover, once bound, genistein stimulates the receptor what is reflected in the increased expression of estrogen–responsive gene (i.e. pS2 gene) as shown in MCF–7 human breast cancer cell line (27,28). This genistein's activity can be abolished in the presence of a classical antiestrogen, tamoxifen. This is the further evidence to confirm the estradiol–like mode of action of genistein. Nonetheless, there were also reported some effects of genistein which could be considered antiestrogenic. Thus, it has been found that this isoflavone attenuates the action of estradiol–activated receptors because when genistein and estradiol were added together to cultured MCF–7 cells, the stimulation effects were lower than when each compound was added alone (28). In addition, prolonged exposure of MCF–7 cells to genistein resulted in a decrease in ER level what consequently led to diminished response to estradiol.

Summing up, it can be seen that genistein can act *via* ER–mediated pathways. Some discrepancies that exist are, however, understandable since genistein can only imitate estrogens to certain extent owing to differences in chemical structure.

### 3.5. Genistein as an inhibitor of proteins involved in multidrug resistance of cancer cells

Multidrug resistance (MDR) in cancer cells is most often associated with overexpression of Pgp (P–glycoprotein) or MRP (multidrug resistance protein) – cellular pumps catalyzing cytotoxic drug efflux from the cells. Pgp and MRP belong to the broad class of transport proteins that utilize ATP and hence they are called ABC–transporters (ATP binding cassette) (29). Genistein has been shown to inhibit their functions, what restores drug accumulation in the resistant cell. The isoflavone was primarily suggested to be specific against MRP activity without affecting Pgp (11), but recent

findings indicate that it can also block Pgp–mediated drug efflux (30). In short, genistein capacity to affect ABC–transporters is ascribed to the competition with ABC substrate (e.g., transported drug) or to competition with ATP or even to direct interactions with the transport proteins what might change their activities.

### 3.6. Genistein interactions with other potential target proteins

The large interest in genistein among the scientific community yielded a number of other possible targets for its action in the mammalian cell. Subsequent new proteins have been proposed to be involved in genistein–mediated effects at the molecular level. One of such proteins is the ribosomal S6 kinase (RS6K). This enzyme is activated in response to many growth factor stimuli (e.g., EGF) and subsequently phosphorylates ribosomal S6 protein, a mandatory step for protein synthesis. Linassier et al. have reported that genistein inhibits EGF–stimulated RS6K activity both in whole cells and in cell–free experiments with extracted kinase (31).

Another clues for a plausible mechanism for genistein's action came from studies on stress genes expression in cancer cells. The key protein regulating this process is a nuclear factor–Y/CCAAT binding factor. The protein normally binds to an important genetic motif in DNA and triggers the stress genes. Stress genes encode for glucose–regulated proteins and heat shock proteins which were shown to protect tumor cells against apoptosis. Zhou et al. discovered that treatment of cells with genistein converted Y/CCAAT binding factor into nonbinding transcriptionally inactive form. The resulting decrease in the expression of stress genes has been suggested as a mechanism of genistein anticancer effects (32).

The proposed alternative mechanism of genistein action concerns its impact on the cellular signaling pathway involving transforming growth factor beta (TGFB). TGFB is a protein which blocks cell growth at the $G_1$/S boundary of the cell cycle. Genistein has been found to augment TGFB–dependent intracellular signaling by up–regulation of TGFB, either by increased synthesis or decreased degradation (33).

Another molecular target of genistein may be a membrane protein forming chloride channel, termed cystic fibrosis transmembrane conductance regulator (CFTR). The mutant form of CFTR, ΔF508CFTR, does not operate properly and is the cause of a serious disease cystic fibrosis. It was found that genistein can enhance activity of CFTR probably by direct binding to the protein what results in prolonging the opening times and in increased chloride currents (34). Furthermore, in the presence of genistein, the mutant protein, ΔF508CFTR, could also be readily activated and its physiological function could be partially restored (35).

Genistein has also been reported to inhibit aromatase and type I 17B–hydroxysteroid oxidoreductase, enzymes responsible for converting peripheral androgens to estrogens and estrone to estradiol, respectively (36). This inhibition may contribute to estrogen–like activity of genistein observed in humans and animals.

### 3.7. Structure–activity relationship of genistein

By comparing biochemical actions of genistein with those of related (iso)flavonoids (see Table 2 and Figure 1) in various experimental models, one could evaluate which motif of genistein structure is responsible for its respective property. Zawa et al. investigated estrogenic and antiproliferative effects of genistein, equol, quercetin and kaempferol by using MCF–7 breast cancer cell line cultured *in vitro*. The results obtained revealed that equol is near equipotent to genistein as an estrogen agonist but much less growth inhibitory. By contrast, quercetin and kaempferol were much weaker estrogen agonists but were nearly equipotent to genistein as growth inhibitors. Furthermore, it was also suggested that the 4'–hydroxy position on the C ring and its spatial orientation with the 7–hydroxy position on the A ring are primary responsible for the estrogenicity of isoflavonoids. Indeed, the 4'–hydroxy group seems to be the most important structural requirement for estrogenic activity of genistein because when this substituent is methylated (i.e. biochanin A) or its position is altered by shifting the phenolic C ring from 3– to 2– position binding on the pyran ring (i.e. genistein→kaempferol), ER binding and estrogenicity are reduced 10–fold. Moreover, when the phenolic C ring of kaempferol is oxidized to a catechol (i.e. quercetin), ER binding is reduced another 10–fold. As regards the antiproliferative effects of genistein and related compounds, 5–hydroxy group and 4–ketonic oxygen seem to be necessary for (iso)flavonoids to be growth inhibitory (36).

In order to clarify the structure–activity relationship of genistein as a PTK inhibitor, Ogawara et al. have compared its activity against EGF–receptor with those of several other isoflavonoids (daidzein, prunetin, biochanin A, genistin) and flavonoids (quercetin, kaempferol, apigenin, flavone, acacetin). Prunetin, kaempferol and quercetin exhibited

Table 2. Some of the most important biochemical properties of genistein and structurally related compounds.

| Property / Compound | Estrogenic activity | PTK inhibition | Topo II inhibition | Inhibition of phosphatidylinositol turnover | Antioxidant activity | Antiproliferative activity |
|---|---|---|---|---|---|---|
| **Genistein** | + | + | + | + | + | + |
| Genistin | | – | – | | | – |
| Daidzein | + | – | | – | + | +/– |
| Equol | + | – | | | +/– | – |
| Biochanin A | – | – | – | | – | + |
| Prunetin | | + | – | | – | + |
| Quercetin | – | + | – | + | + | + |
| Apigenin | | – | – | | +/– | + |
| Kaempferol | – | + | | | | + |
| Flavone acetic acid | | | – | | | |
| Acacetin | | – | | | + | + |
| Flavone | | – | | | | + |

+ compound exhibits activity
+/– activity less pronounced than that of genistein or some discrepancies exist in the literature
– compound hardly does or does not exhibit activity

appreciable inhibitory activity, not much less than genistein itself. The inhibitory activity decreased drastically either by the removal of a hydroxy group from 5 position (flavone and daidzein) or by the addition of a methoxy group to 4' position (biochanin A and acacetin). Addition of a methoxy group at the 7 position (prunetin) also reduced the inhibitory activity. Especially a bulky group at 7 position such as a O–glucose (genistin) completely abolished the activity. These results indicated that a hydroxy group at 5 position is essential for inhibitory activity and that at 7 and 4' positions is necessary for full expression of the activity (37).

As regards the ability of genistein to inhibit topo II, it appears to be unique among (iso)flavonoids. Closely related compounds such as genistin, biochanin A, prunetin, apigenin, flavone acetic acid and quercetin have been found to be inactive (10).

To summarize, genistein has two principal biochemical properties related to its chemical structure. The first one is its estrogenicity – common to other isoflavonoids having a free hydroxy group at positions 4' and 7. The second property is the ability of genistein to inhibit some of cellular enzymes – what may result in antiproliferative or cytotoxic effects. For this inhibition, the intramolecular hydrogen bonding (formed by 5–hydroxyl and 4–ketonic oxygen) seems to be the most important feature of the genistein structure. This bond is very strong (according to *ab initio* calculations its energy is ca. 15. kcal/mol (38)), creates an additional six–membered heterocycle ring in the genistein molecule and makes it more hydrophobic (although genistein has one additional hydroxy group compared to its analogue daidzein, genistein is more hydrophobic (9)). The differences in hydrophobicity may also partially contribute to explain unique genistein's biochemical and biological properties among (iso)flavonoids.

## 4. Mechanisms of genistein action at the cellular level and in the experimental animal models

Multidirectional biochemical activity of genistein *in vitro* parallels numerous effects of this compound that can be observed in cultured cells and in various experimental animal models.

### 4.1. Induction of apoptosis

Rapidly proliferating cancer cells treated with genistein usually undergo apoptosis. This phenomenon can be ascribed to the following events in cellular life induced by the isoflavone:

- Inhibition of topo II and causing DNA strand breaks,
- Cell cycle derangements,
- Inhibition of protein tyrosine kinases since these enzymes are known to protect cells against apoptosis,
- Interfering with phosphatidylinositol cascade of the signal transduction,

- Converting of Y/CCAAT binding factor into transcriptionally inactive form, what reduces the stress genes expression.

### 4.2. Induction of cellular differentiation

One of the major strategies currently being used in the fight against cancer is the use of noncytotoxic concentrations of agents that promote terminal differentiation (and hence inhibit proliferation) of human tumor cells. Numerous cancer cell lines treated with genistein undergo differentiation which may be followed by apoptotic cell death. For example, HL–60 cancer cells (human promyelocytic leukemia) acquired granulocytic or monocytic markers. K–562 (erythroid leukemia) cells started to produce hemoglobin – a marker of erythroid differentiation. In melanoma cells the formation of dendrite–like structures was observed as well as the increase in melanin content and activity of tyrosinase – all changes indicative of maturation (39). In another experiment, genistein–treated neuroblastoma cells ($N_2A$) exhibited morphological features of differentiation evidenced by development of dendritic extensions (40). A number of genistein activities have been proposed to account for this phenomenon:

- Inhibition of topo II and causing DNA strand breaks since other topo II inhibitors have also been shown to induce cancer cells differentiation (23,39),
- Inhibition of PTKs because decreased activity of these enzymes has been implicated in the differentiation of various cell types (39),
- Inhibition of phosphatidylinositol turnover what influences cellular signal transduction program (26).

### 4.3. Inhibition of cell proliferation

In cancer and nontransformed cell lines cultured *in vitro* proliferation is usually stimulated with serum or growth factors. They act by membrane bound receptors which upon activation originate cascade of mitogenic signals branching in cytoplasm and reaching nucleus. Genistein has been shown to attack different signal transduction enzymes what results in the decreased rate of cell proliferation. On the other hand, genistein treated cells stop proliferating due to cycle derangements, apoptosis or differentiation. For the majority of cell lines, the $IC_{50}$ value of genistein ranges from 5 to 40 μM/l (41). Importantly, this compound is not cytotoxic to cells at its $IC_{50}$ values. In fact, its effect on cell growth is fully reversible in most cases. Generally, genistein growth inhibitory effects are better pronounced in leukemic cell lines than in cell lines derived from solid tumors (13).

Cell proliferation studies may also serve as a good example to present multidirectional activity of genistein in the live cell. In estrogen receptor positive cells, such as MCF–7 breast cancer cell line, genistein at low concentrations (10–1000 nM/l) stimulates proliferation (maximal effect about 3–fold over that of control) while at higher concentrations (>10 μM/l) the precipitous drop in the cell growth is observed (28,36). This biphasic growth response to increasing concentrations of genistein is probably the result of at least two mechanisms by which genistein can alter cell proliferation. One mechanism stimulates cell proliferation at low genistein concentrations and is likely to be mediated *via* the estrogen receptors. The other mechanism, which is antiproliferative, is active at high concentrations of the compound and is likely to be mediated *via* inhibition of tyrosine phosphorylation or *via* other cellular mechanisms such as inhibition of PI/PIP kinases, topo II, etc. Thus, genistein while mediating its actions *via* ER apparently also cross–talks with other ER–independent cellular mechanisms that can override growth–stimulatory effects from low genistein concentrations. In cancer cell lines without functional ER system the isoflavone only inhibits proliferation at higher concentrations, showing no effects at lower levels in the growth medium (9,27,28,36).

### 4.4. Alterations in the cell cycle progression

Cell cycle is a sequence of events in cellular life leading to cell division and proliferation. This process is controlled by a variety of enzymes and some of them can be affected by genistein directly or indirectly. Topo II and PTKs are known to be involved in cell cycle progression to large extent. So it is not surprising that genistein being both topo II and PTKs inhibitor can modulate cell cycling (39). Additionally, this isoflavone has been shown to regulate cell cycling also *via* augmenting TGFB–dependent signaling – what leads to a block at $G_1$/S boundary as was shown in normal human mammary epithelial cells (33). In MCF–7 breast tumor cells, genistein exerted a strong inhibitory effect on the progression through two critical points of the cell cycle control, namely G1–S transition and M–phase entry (21). In Jurkat T–leukemia cell line cell cycle analysis revealed G2/M arrest at low genistein concentrations (18–37 μM/l), whereas at higher doses (74–111 μM/l) there was also a perturbation in the S–phase progression (20). Traganos et al. have investigated the impact of genistein on cell cycle kinetics both in normal human leukocytes and in leukemic cell lines (MOLT–4 and HL–60). At concentrations up to 74 μM/l, genistein in

short–term exposures (4–8 h) did not effect proliferating lymphocytes but caused perturbations in S and G2 phase in cancer cells. A stathmokinetic experiment using MOLT–4 cells revealed that genistein at 18.5 μM/l suppressed cell exit from S to G2 phase with a terminal point of action at, or near to, the S–G2 border. Cell progression through the early point of G1 phase (G1A, characterized by postmitotic chromatin decondensation) was also suppressed, whereas cell advancement through the reminder of G1 phase was not markedly affected. Longer exposure (24 h) of proliferating lymphocytes to 74 μM/l led to S–phase arrest, while similar treatment of leukemic cells caused cell arrest in G2 phase and an increase in the number of cells entering cell cycle at higher DNA ploidy (42).

In conclusion, it can be seen that genistein can block cell cycling at various points and each phase of cycle can be disturbed by the compound. The final point, where genistein–induced blockage occurs, seems to depend on several factors such as: the concentration of genistein, the time of exposure, the type of used cells, and the experimental conditions.

### 4.5. Antioxidant effects

Reactive oxygen species (ROS) are known to play an important role in mutagenesis, carcinogenesis and particularly in tumor promotion. These compounds are also produced by activated cells of the immune system and may be the cause of tissue damage. Additionally, peroxidation processes have been implicated in atherosclerosis etiology, especially the oxidative modification of LDL (low density lipoprotein). Genistein can inhibit both the priming events necessary for high level ROS production or can directly inhibit agonist–stimulated ROS production (13). These antioxidant effects of genistein have been demonstrated in a number of experiments: In HL–60 cancer cells and human polymorphonuclear cells cultured *in vitro*, $H_2O_2$ formation was stimulated by a phorbol ester. Genistein inhibited $H_2O_2$ production in a dose–dependent manner with a 50% inhibitory concentration of 14.8 and 30.2 μM/l, respectively. In addition, this isoflavone moderately inhibited superoxide anion formation by HL–60 cells and scavenged exogenously added $H_2O_2$ under the same conditions as in cell culture (43). Further evidence for genistein antioxidant effects was from *in vitro* studies performed by Kapiotis et al. (44) They have found that the compound was able to inhibit the oxidation of LDL in the presence of copper ions or superoxide/nitric oxide radicals. Additionally, bovine aortic endothelial cell– and human endothelial cell–mediated LDL oxidation was also inhibited in the presence of genistein. So it is clear that this isoflavone is effective in protecting LDL both in cell–free and cell–mediated lipoprotein–oxidating systems. Moreover, when cultured human endothelial cells were challenged with already oxidized lipoproteins, it appeared that genistein effectively protected the vascular cells from damage by these lipoproteins. Thus, genistein can act as an LDL antioxidant and a vascular cell protective agent *in vitro*. Further studies have demonstrated that genistein can also function as an antioxidant *in vivo*. When this isoflavone was administered orally with the diet (250 ppm) for 30 days, it significantly increased the activity of antioxidant enzymes in various organs of Sencar mice. The activity of catalase was enhanced in small intestine, liver and kidney, the activities of superoxide dismutase and glutathione peroxidase increased in skin, and the activity of glutathione reductase was elevated in skin and small intestine (45).

The aforementioned data show that genistein can function as an antioxidant both in cell–free systems, in intact cells, and in mammalian organism. This activity may serve to explain some of the advantageous biological properties of the compound such as anticancer or antiatherogenic effects, for instance.

### 4.6. Antiangiogenic effects

Angiogenesis that is the generation of new capillaries is a physiologically important process, among other factors, involved in cardiac arrest protection in developed atherosclerosis. However, possibly the most clinically important manifestation of pathological angiogenesis is that induced by solid tumors. Once a tumor grows beyond a millimeter it must foster the growth of new blood vessels into it to be properly supplied with oxygen and glucose. Thus, it is clear why antiangiogenic compounds attract so much attention as new anticancer agents. It has been demonstrated that genistein is the most potent amongst several plant derived inhibitors in preventing angiogenesis. Fotsis et al. have found that genistein was able to inhibit the proliferation of vascular endothelial cells and *in vitro* angiogenesis at half maximal concentrations of 5 and 150 μM/l respectively (46). Antiangiogenic activity of genistein has also been documented in *in vivo* studies in mouse xenograft of various cancer cells. For example, syngeneic C57BL/6 mice were inoculated subcutaneously with bladder carcinoma cells, and tumor growth was quantitated. When these mice received genistein (i.p., 50 mg/kg body weight/day), tumor size

was reduced and further microvessel quantitation showed that vessel density was also decreased, compared with control animals (47). Similar results indicating antiangiogenic activity of genistein were obtained when nude mice were inoculated with MDA–MB–231, human breast carcinoma cell line. In this case, genistein decreased neovascularization in tumor tissue as well (48). Another line of evidence comes from experimentally induced angiogenesis in animals. For example, corneal neovascularization was induced in NZW rabbits by the use of methylcellulose discs loaded with basic fibroblast growth factor. Blood vessels grew from the limbus toward the pellet and were quantified under the microscope. Genistein, when injected subconjunctivally (40 μg/day), showed significant reduction of blood vessels at the limbus and vascularized area in the eye was also decreased (49). Moreover, topical treatment of genistein eye drop (5 mg/ml) turned out to be effective in preventing extensive neovascularization of cornea induced by chemical cauterization in rats (50).

In short, it can be seen that genistein is a potent antiangiogenic substance. Therefore, it may find important applications in the treatment of solid tumors as well as other diseases characterized by progressive neovascularization.

### 4.7. Reversing of multidrug resistance of cancer cells

Multidrug resistance is the simultaneous resistance to several types of commonly used anti–neoplastic agents and it leads to the failure of cancer chemotherapy mainly owing to decreased drug accumulation in resistant cells. There are at least four biochemical mechanisms that can confer MDR (overexpression of Pgp or MRP, alterations in topo II, overexpression of lung resistance protein). The one mediated by MRP leads to increased drug efflux out of cell or/and drug sequestration inside cell what ultimately reduces the access of cytotoxic drugs to their targets. Genistein has been found to be an inhibitor of MRP and was widely used to dissect molecular pathways of MDR. In several MRP–overexpressing cancer cell lines genistein treatment resulted in the increase of drug accumulation due to the inhibition of outward, ATP–dependent drug transport across the plasma membrane (29). Additionally, genistein was able to block MRP–mediated drug sequestration in intracellular compartments – the process responsible for protecting nucleus against anti–neoplastic agents. In membrane vesicles containing high levels of MRP, genistein has been shown to be a competitive inhibitor of cytotoxic drug transport by the protein (51). In spite of some reports suggesting genistein to be a specific MRP inhibitor, recent findings seem to deny these concept because genistein has also been revealed to block Pgp. Castro et al. have reported genistein to interact with Pgp and inhibit Pgp–mediated drug efflux. They concluded that genistein may affect both Pgp and MRP function (30). Such controversies as regards the genistein effects on the transporters are not entirely unexpected since the compound holds a wide range of biochemical activities and only some of them may be relevant depending on employed cell type or experimental system. To explain the mechanisms of genistein–mediated reversal of MDR, a number of mechanisms have been proposed in the literature. First of all, genistein is supposed to interact with drug transporting protein directly or to compete with ATP or drug molecule. Subsequent investigations revealed that genistein depleted cellular ATP level what certainly can contribute to reduction of MRP and Pgp biological potency. Furthermore, in genistein treated MDR cancer cells, the cellular glutathione level was significantly decreased. This tripeptide plays a central role in the cellular drug detoxification processes, hence genistein–induced glutathione depletion has been proposed as another mechanism responsible for restoring drug sensitivity (52).

In conclusion, initial interest in genistein as a MDR modifier raised from its ability to block transport proteins conferring MDR. So genistein (besides hundreds of other chemical compounds) was used to explore or to overcome this phenomenon *in vitro*. Unfortunately, concentrations of genistein required for reversing MDR were always very high (ca. 200 μM/l), far above $IC_{50}$ for the growth inhibition. Therefore, possible genistein application as a MDR modifier *in vivo* is rather out of the question.

### 4.8. Estrogenicity of genistein

One of the most remarkable properties of genistein well recognized in various animal species is its estrogenic activity. Along with a large number of other isoflavones, genistein belongs to the class of phytoestrogens, i.e., plant derived estrogens. Phytoestrogens have been shown to decrease fertility of sheep grazing on subterranean clover, rabbits fed on soybean hay, captive cheetahs fed diets enriched with soybean protein, and desert quail feeding on desert brush. Moreover, a decrease in reproductive performance has also been noted in certain strains of female rats fed either soybean–based diet or diet supplemented with genistein. These observations have been at-

tributed to estrogenic effects produced by dietary phytoestrogens (27), and especially by genistein (6,7). Direct estrogenic activity of genistein has been then confirmed in series of *in vitro* and *in vivo* experiments.

Various cancer cell lines which possess functional ER system increase proliferation rate in response to estradiol treatment. In such cells genistein at low concentrations can mimic the action of estradiol and stimulate cell growth. Furthermore, these results can also be reproduced *in vivo* in the following experimental model: Athymic nude mice were implanted subcutaneously with ER positive MCF–7 breast cancer cells and were fed standard diet (negative control) or a diet supplemented with genistein. Positive control group received a new subcutaneous estradiol pellet. Tumors were larger in estradiol and genistein–treated groups than those in negative control group, indicating that genistein was able to act as an estrogen agonist *in vivo* (27). In another experiment, the immature mouse uterine weight method was employed to investigate this genistein property. The isoflavone was administered either subcutaneously or orally and after four days the uterine weights were measured. Genistein stimulated the uterine growth in a dose dependent manner and its activity was evaluated to be approximately equivalent to 1/50,000 the activity of diethylstilbestrol – a synthetic estrogenic compound (53). Similar results were obtained with athymic mice which were ovariectomized to be devoid of natural estrogens. In this case genistein occurred to be able to substitute for endogenous estradiol since the isoflavone caused increase in mouse uterine weight as well as stimulated mammary gland growth (27).

Estrogenic activity of genistein may also be demonstrated in its effects on the bone condition. For example, femoral–metaphyseal tissues obtained from elderly female rats were cultured *in vitro* in the presence of bone–resorbing factors: LPS (lipopolysaccharide), PGE2 (prostaglandin E2), or PTH (parathyroid hormone). Genistein at concentrations 0.1–10 μM/l completely inhibited bone degradation as measured by bone calcium content, in which it closely imitated the action of estradiol. In addition, this protective effect of genistein was abolished in the presence of classical antiestrogen tamoxifen (54). Similar conclusions were drawn from the studies on anabolic effects of genistein on bone metabolism *in vitro*. Like estradiol, genistein enhanced bone formation as evidenced by the increase in calcium and DNA content and increase in alkaline phosphatase activity, what again could be blocked by tamoxifen (55). Estrogen–like effects of genistein have also been shown *in vivo* in animal model of osteoporosis. Treatment with the compound of ovariectomized female rats resulted in an equivalent % retention of bone mineral mass as a physiological doses of estradiol (56).

### 4.9. Inhibition of osteoclastic function

Osteoclast are macrophage derivatives which mediate physiological and pathological bone degradation and osteoporosis results when the rate of osteoclastic bone resorption exceeds bone formation. Recent studies have revealed that genistein was a very potent osteoclast inhibitor and substantially decreased bone resorption in *in vitro* experiments. Genistein–treated osteoclasts in bone tissue cultures lost their bone degradation potency as measured by decrease in: lactic acid production, medium glucose consumption and acid phosphatase activity. Importantly, all these phenomena occur at relatively low genistein concentrations ranging from 0.1 to 10 μM/l. According to the results obtained by Yamaguchi et al. (54), the effect of genistein to inhibit bone resorption was due to estrogen–like action of the isoflavone. Apart from this action genistein has also been suggested to suppress osteoclast function through inhibition of PTKs. It may be the case since this bone degrading cells are unusually dependent on tyrosine kinase activity. At the same time osteoclastic protein synthesis was significantly inhibited only at genistein concentrations three fold greater than those inhibiting bone resorption, what suggests a low toxicity of the isoflavone (57).

### 4.10. Immunosuppressive activity

Besides other model PTK inhibitors, genistein has been widely used in numerous *in vitro* experiments with various types of white cells. These experiments were aimed rather to gain insight into the role which PTKs play in the activation process of leukocytes, than to explore the immunosuppressive properties of genistein. Nonetheless, they have revealed both the pivotal role of tyrosine phosphorylation in proper leukocyte function and the potency of genistein as a novel immunosuppressant. For example, Stewart et al. have shown that genistein prevents activated killer T (AK–T) lymphocyte–mediated tumoricidal activity. In their studies, AK–T lymphocytes destroyed tumor cells and this process was blocked by genistein. The lack of tumoricidal activity was not due to reduced lymphocyte binding to target tumor cells but was associated with the abrogation of granule exocytosis delivering the „lethal hit" by AK–T lymphocytes (58). In another study, genistein attenua-

ted the adherence of normal blood neutrophils, lymphocytes and monocytes to monolayers of human umbilical vein endothelial cells (59). The adherence ability of leukocytes is one of the important symptoms of their proper activity.

Stimulated T lymphocytes transform from passively transported cells in the circulation in the vascular system to actively migrating cells during extravasation. This motile behavior could be blocked if T lymphocytes were pretreated with genistein (60). In addition, genistein has been shown to inhibit the natural killer (NK) lymphocytes activation induced by LPS or fixed–bacteria (61). It has also been reported that the signal transduction cascade initiated by T–cell receptors may be attenuated by genistein what counteracts antigen–specific activation of T lymphocytes (62).

Another series of studies were aimed at investigating the efficacy of genistein as an immunosuppressive drug both *in vitro* and *in vivo*. For example, Atluru et al. compared the impact of genistein with cyclosporin A (CsA) on human T lymphocytes (63): CD28 monoclonal antibody was used to stimulate lymphocyte proliferation, IL–2 production and expression of IL–2 receptor. All these activities were inhibited by genistein (with no toxic effects on T–cells) while CsA was inactive. So the authors concluded that genistein blocked CsA–resistant pathway of T–cell activation and therefore the combined usage of these two agents may provide better immunosuppressive results. The effects of genistein on normal human lymphocytes has also been explored by Traganos et al. (42) Phytohemagglutinin–stimulated lymphocytes transited from phase G0 to G1, entered the cell cycle and started to proliferate. Proliferating lymphocytes were not very susceptible to genistein treatment if compared to proliferating leukemia cells such as MOLT–4 and HL–60. On the contrary, the mitogen–induced transition from G0 to G1 in lymphocytes was extremely sensitive to inhibition by genistein. The 50% inhibition concentration was only 6 μM/l, what suggests that genistein might be a very potent immunosuppressant as it effectively counteracts activation of lymphocytes. This concept has been recently corroborated *in vivo* in the animal model: Fiedor et al. have used a synthetic genistein–piperazine complex (with genistein as the active moiety) to investigate its effects on survival of pancreatic islet allografts (64). Diabetic Lewis rats were recipients of islet allografts and then they received injections of 3 mg/kg body weight of the complex, in various regimens. The drug significantly prolonged the survival of transplanted islets what was measured by the period of normoglycemia and histopathology sections.

The aforementioned investigations indicate the potential use of genistein in the prophylaxis and treatment of allograft rejection, additionally they stress genistein low toxicity and distinct mechanism of action from the currently used immunosuppressants. The majority of studies suggest inhibition of PTKs as mechanism to explain genistein activity in leukocytes, but the role of genistein–induced inhibition of topo II is also worth consideration.

### 4.11. Mast cell stabilization

Mast cells play a central role in the development of allergic diseases. Their cytoplasm is abundant in granules filled with inflammatory mediators which are released following mast cell degranulation. This process is usually initiated by allergen (antigen) binding on the cell surface what triggers cascades of biochemical signals transmitted through the plasma membrane into the interior of the cell. These biochemical pathways of stimuli signal transduction involve tyrosine phosphorylation and phosphatidylinositol turnover. Hence no wonder that genistein, being the inhibitor of both PTKs and PI/PIP kinases, can stabilize mast cells, that is to block their degranulation. This genistein activity has been shown *in vitro* in a model of bronchial asthma: The isoflavone attenuated antigen–induced anaphylactic contraction of the isolated bronchi of guinea pig (14) as well as inhibited tracheal contraction in a similar experimental model (65). These inhibitory effects of genistein were due to mast cell stabilization in the bronchi and trachea. Moreover, the release of histamine and peptidoleukotrienes from chopped lung preparations of guinea pig was also decreased in the presence of genistein, what indicated the inhibition of mast cell degranulation in the lung tissue. Based on these findings genistein was suggested to have a therapeutic potential in the treatment of allergic diseases.

## 5. Genistein in prophylaxis and therapy

### 5.1. Toxicity of genistein

The question of genistein toxicity should be considered in two categories. The first one concerns genistein as a chemopreventive compound for the long–term administration. In this case nearly five millennia of soy consumption in southeast Asia seem to prove that chronic exposure to low amounts of genistein is not toxic to humans (although other species may be susceptible e.g., sheep and cheetahs). Thus, heavy soy–consumers have

serum genistein concentration in the range of 1–5 μM/l (66), mostly as glucuronide and sulfate conjugates. Such level of genistein has not been associated with any unfavorable effects, quite the contrary, it is health beneficial. Similar conclusions have been drawn from studies on animals. For example, six months of a diet enriched with soy proteins had no adverse effects on reproductive system of prepubertal rhesus monkeys as evaluated by reproductive hormone concentrations and organ weights at necropsy (67).

The other issue for consideration is genistein toxicity as a potential drug administered at higher doses. This subject has not been investigated profoundly yet, but some data exist and they suggest genistein not to be toxic in this case, too. Thus, no acute toxicity was observed after intraperitoneal injection of 100 mg/kg of body weight of mice and even at 500 mg/kg in another experiment (3,4). If one assumes that genistein equilibrates with total body water (66) then such doses, immediately after injection and disposition, would give serum concentration about 620 and 3080 μM/l, respectively while e.g., for anticancer activity only 5–40 μM/l (90) is required in most of *in vitro* experiments. Toxicity of the isoflavone has also been assessed on occasions of various studies aimed to explore a particular genistein activity in laboratory animals. For example, genistein was administered to female rats at doses of 60 mg/kg body weight to investigate its impact on the bone loss. During 30 days of such regimen, weights of treated and control animals were indistinguishable, indicating no toxicity (57). In another experiment three high doses (500 mg/kg, every second day) of genistein given for prepubertal female rats were without significant toxicity to endocrine/reproductive system of the animals (68).

To sum up, low doses of genistein have certainly no toxic effects on humans as evidenced by the history of soy consumption. The impact of higher doses, those for anticancer action, is not well recognized yet but available literature data suggest genistein to be not toxic in such cases, too.

### 5.2. Cancers

Three distinct investigative pathways led to the discovery of genistein as an anticancer compound. Firstly, epidemiological data supported by experimental studies strongly emphasized genistein to be a chemical constituent responsible for the antitumor properties of soyfoods (41). On the other hand, in 1981, Japanese investigators searching for novel anticancer substances isolated from the fermentation broth a compound that induced differentiation of erythroleukemia cells and called it differenol A (3). Subsequent studies revealed it to be identical with genistein. Another pathway towards genistein led through the investigations of specific tyrosine kinase inhibitors (8). Such inhibitors were expected to exhibit antitumor activity since it had been found that pathological phosphorylation of tyrosine residues of various proteins is involved in malignant transformation. All these discoveries were followed by numerous studies aimed at gaining more insight into genistein's mode of action. In experimental models with laboratory animals, genistein has been proved to be a potent anticancer agent due to several activities which made it a promising compound both in cancer prophylaxis and therapy. First of all, estrogenic properties of genistein seem to play a basic role in hormone–dependent cancer prevention both in women and men. As regards the female reproductive tissue cancers, this presents a paradox because estrogens are well–recognized promotors of such types of cancers. Nonetheless, epidemiological studies have proved that Asian women who consume soyfoods (and hence genistein) as a dietary staple have a 5–10 lower risk of developing breast and endometrial cancers than women living in Western world (36). Additionally, when the tumor does occur it tends to be more differentiated and follow a better clinical course. One of the possible explanations of this paradox may lay in timing of genistein administration. If an estrogenic substance is administered before maturation of e.g., mammary gland and before initiation with mammary carcinogen, then the number of tumors will be reduced, owing to the effect of estrogen in causing mammary gland maturation (27). Thus, chronic exposure of target tissues to genistein from daily consumption of soyfoods leads to more reproductive tissue maturation, reduces cell proliferation in mammary gland, thus reducing the possibility of cancer initiation by exogenous carcinogen. This concept has been corroborated in animal models where a high–soy diet as well as genistein itself prevented and inhibited the growth of carcinogen–induced breast tumors (66). When genistein was administered subcutaneously to prepubertal rats (three doses of 500 mg/kg, every second day) followed by the initiation of mammary tumors with DMBA (7,12–dimethylbenz(a)anthracene), a reduction in incidence and number of tumors per animal was noted in relation to control group. This genistein exposure during the prepubertal period suppressed the development of chemically–induced tumors by causing early mammary gland development and cell differentiation (68). Proposed alter-

native mechanism by which genistein, acting as a weak estrogen, can prevent breast cancer concerns its influence on women endocrine system. When ingested it may serve to downregulate the hypothalamus and pituitary and thereby reduce the ovarian synthesis of estrogens. In premenopausal women, a one month genistein–containing soy protein diet increased the length of the follicular phase and delayed menstruation (69). Another clinical study with six healthy premenopausal females showed that soymilk ingestion (100 mg genistein/day) reduced the level of estradiol and progesterone and increased the menstrual cycle length (70). Lower levels of ovarian hormones decrease the risk of breast cancer. Proposed alternative mechanism by which genistein can prevent the occurrence of life–threatening breast tumors includes the inhibition of subsequent stages of cancer progression which result in invasion and metastasis (71). Besides prevention, efficacy of genistein has also been demonstrated in the treatment of established model mammary tumors in animals. For example, nude mice were inoculated subcutaneously with human breast carcinoma cells either ER–positive or ER–negative and the tumor growth was quantified. The volume of tumor was significantly decreased when the animals received genistein. This growth inhibition was due to combination of direct effects on tumor cells (e.g., stimulation of apoptosis) and indirect on tumor neovasculature (inhibition of angiogenesis, decreasing the level of vascular endothelial growth factor and transforming growth factor–beta1) (48). To summarize, these data show that genistein has an appreciable activity against breast cancer and is likely to become an innovate therapeutic agent for this disease.

Epidemiological findings have also shown that in countries with high soyfood consumption the mortality from prostate cancer is low, despite the same incidence of latent and small or non–infiltrative prostatic carcinomas as in Western world (72). The therapeutic effect of estrogens in prostatic cancer suggests that soy constituent responsible for this protection is probably genistein. Indeed, the isoflavone through its estrogen–like action may postpone the occurrence of metastatic prostate cancers but unlike estrogens it does not show feminizing syndromes in males. Moreover, *in vitro* studies have shown that prostatic cancer cells loose their invasive potency when grown in the presence of genistein (73) and this may further contribute to protective effects of the compound. In another experiment the impact of genistein was investigated on human–patient benign prostatic hypertrophy (BPH) and human prostate cancer tissue cultured *in vitro*. The growth of BPH and cancer tissue, measured by incorporation of $^{3}$H–thymidine, was decreased by genistein in a dose–dependent manner (74). These *in vitro* findings were reinforced by a study on animal models: Lobund–Wistar rats that are inherently susceptible to prostate–related cancers were fed a soy isoflavone supplemented diet being the source of genistein. The incidence of chemically–induced cancer was reduced and the disease–free period was prolonged in the isoflavone ingesting group relative to control animals (75). Taken together epidemiological reports as well as *in vitro* and *in vivo* data provide a rationale for further genistein development as a chemotherapeutic agent for prostate cancer treatment.

High soy intake has also been found to prevent colon cancers (41). One of the soy constituents responsible for this protection may be genistein. This concept is based on the study in which genistein was used as an chemopreventive agent in a chemically–induced colon carcinogenesis model. Genistein was tested for its ability to inhibit aberrant colon crypts in the colon of F344 rats that had been treated with azoxymethane. The isoflavone was administered in the diet at doses of 75 and 150 mg/kg from one week before to four week after the first carcinogen dose. The genistein–treated group had a significant reduction in the number of foci per colon (12). This result clearly emphasized the role of genistein as a potential chemopreventive drug and partially served to explain the low rate of colon cancers in heavy soy consumers.

Despite the fact that research interest in genistein is mostly focused on the subject of cancer prevention, its importance in the cancer treatment is constantly increasing since subsequent studies reveal genistein's efficacy against model tumors implanted into laboratory animals. Thus, apart from the above–mentioned breast cancers the *in vivo* growth of other cancerous cells may be also inhibited by genistein. This has been noted for bladder cancer cells inoculated subcutaneously into syngeneic mice (47) as well as for melanoma cells in a similar experimental model (76). To explain genistein–mediated growth inhibition of tumors *in vivo* several, *in vitro* explored, mechanisms can be proposed. They include antiproliferative effects, apoptosis, cell differentiation, antioxidant activities and inhibition of tumor neovascularization.

Unique genistein usage as a novel anticancer drug has been demonstrated by Uckun et al. (77). They have conjugated genistein with monoclonal antibody against receptor tyrosine kinase of B–cell

precursor leukemia (BCP). This immunoconjugate turned out to be an extremely efficacious agent in the BCP leukemia treatment. In mouse model of this disease, genistein immunoconjugate was far more potent than any other regimen and, what is most important, its application led to 100% event–free survival from an otherwise invariably fatal leukemia. The authors suggested the usage of the compound in eliminating leukemia cells in patient who have failed conventional therapy.

To sum up, epidemiological data supported by experimental findings provide a rationale for genistein usage in the fight against cancer. Such a battle include both prophylaxis and therapy of developed tumors. Soyfoods or genistein–supplemented diet may constitute the basis for cancer chemoprevention, especially in high risk patients who would potentially benefit from such treatment. In mature, established tumors genistein would have to be administered at multiple higher doses than those needed for the long–term prophylaxis. In chemoprevention health profits of genistein are well recognized. In the case of mature tumors the efficacy of genistein has to be more explored but at present, at this point in time it can be concluded that genistein is a very promising agent mainly due to its low toxicity in which it strongly differs from currently used chemotherapeutics.

### 5.3. Postmenopausal bone lose and osteoporosis

Postmenopausal estrogen deficiency leads to severe consequences including bone loss resulting in osteoporosis. However, women consuming soyfoods and hence exposed to dietary genistein are less prone to the development of this disease (36). Genistein exerts a quite significant estrogenic activity and therefore may be an efficient agent in retaining bone mass. Animal model studies with ovariectomized rats (devoid of endogenous estrogens) have proved that genistein is as active as estrogens in maintaining bone health (56). In tissue cultures *in vitro* genistein, like estradiol, exerts a significant protective activity against experimentally induced bone resorption (54) and furthermore it stimulates osteoblast–mediated bone formation, that is, it shows anabolic effects (55). Moreover, on the basis of a comparative study with model PTK inhibitors, genistein has been reported to block osteoclastic function *in vitro* through tyrosine kinase mechanism. This mechanism has been then proposed to be also relevant *in vivo* suggesting that the effects of genistein on bone health are not limited to agonism at the estrogen receptor. To sum up, whatever the mode of action of genistein is it appears to be a very potent agent in the prevention of osteoporosis. This conclusion can be further supported by the results of a clinical study involving 66 postmenopausal women who received soy isoflavone–enriched diet for six months. Such treatment led to the significant increases in bone mineral content and density in the lumbar spine (78). These health benefits may be, at least in part, ascribed to genistein as it is the predominant and the most active soy isoflavone.

### 5.4. Cardiovascular diseases

Although dietary soy protein is well recognized for its beneficial effects in the promotion of cardiovascular wellness the role which genistein plays is still largely under investigation. Data from *in vitro* experiments indicate genistein to have the potential for antiatherogenic action *in vivo*, however, most of the studies on animals or humans were conducted with soy protein or soy isoflavones but not with pure genistein. Nonetheless, biological effects noted in such studies may be largely ascribed to genistein since it is the most abundant and biologically active soy constituent.

In individuals with conditions such as atherosclerosis and heart disease excessive platelet aggregation leads to clot formation and may increase the risk of heart attack and strokes. Consequently, any factor, which inhibits excessive platelet aggregation, is potentially beneficial. Genistein has been shown to inhibit platelet aggregation *in vitro* (79) but *in vivo* studies with 20 male subjects ingesting soy proteins for 28 days (plasma genistein level ca. 1 μM/l) did not confirm this genistein's activity (80). Nonetheless, this does not rule out the possibility that undetectable inhibition *in vivo* occurs which might slightly contribute to the overall genistein effect.

Evidence is accumulating that genistein may counteract atherosclerosis development by acting as an antioxidant. *In vitro* genistein inhibits LDL oxidation by reactive oxygen species and effectively protects human vascular endothelial cells from damage by oxidized lipoproteins (44). *In vivo* genistein enhances activity of antioxidant enzymes as it was demonstrated in mice (45).

Another line of evidence indicating the importance of genistein as an antiatherogenic agent is from numerous studies on humans and monkeys. These studies have clearly shown that long–term administration of soy isoflavones reduces the risk factors associated with cardiovascular diseases. Thus, 80–mg daily isoflavones (45 mg genistein) over 5– to 10–week period have appreciably improved systemic arterial compliance (arterial elasticity) in menopausal and perimenopausal women

to about the same extent as is achieved with conventional hormone replacement therapy (81). In another study involving 66 postmenopausal women effect of soy isoflavones was investigated on blood lipids. The women ingested 56 or 90 mg of isoflavones per day for six months. Both doses significantly reduced non–HDL cholesterol, increased HDL cholesterol and heightened the level of mononuclear cell LDL receptor mRNA (78). All these changes were health beneficial since they decreased the risk of cardiovascular disease development. Similar conclusions have been drawn from studies on cynomolgus monkeys, where the diet containing soy isoflavones proved to be quite effective in improvement of LDL and HDL cholesterol (82). In addition, high–isoflavone diet has been shown to enhance coronary vascular reactivity in atherosclerotic female macaques and this enhancement could also be provoked by intravenous genistein administration (83).

To sum up, *in vitro* data suggesting the role of genistein in the promotion of cardiovascular wellness are reinforced by animal and human studies. These studies further indicate its potential use in high risk patients such as postmenopausal women who might especially benefit from genistein treatment.

### 5.5. Menopause

Epidemiological findings show that postmenopausal Asian women who consume soy–rich diet have fewer health problems commonly associated with estrogen deficiency such as osteoporosis, cardiovascular disease and menopausal symptoms. It is the case, although they reach menopause earlier and have only about one–half the circulating levels of estradiol and estrone of Caucasian American women (36). These health benefits are ascribed to soy–derived genistein that is believed to substitute for the lack of human endogenous estrogens, mitigating the effects of their absence. In fact, genistein is capable of alleviating the symptoms of menopause including depression, hot flashes, sleeplessness, irritability etc.

### 5.6. Other disorders

The usage of soy–derived products provide a good, safe means for investigating the role of isoflavones and hence genistein in numerous human diseases. Thus, consumption of soy–protein based beverage appeared to give a drastic improvement in the familial chronic nose bleed disorder, a genetic disease involving mutations in TGFB receptor complex. Genistein has been found to act in cell *via* TGFB–dependent mechanism and therefore the health benefits of the used diet might be ascribed to genistein. Patient who ingested such soy product for one week had drastically reduced the incidence of nosebleeds (33).

Genistein is also supposed to be helpful in cystic fibrosis, the most common deadly genetic disease in Caucasian race. Cystic fibrosis is caused by inherited mutations in a gene termed cystic fibrosis transmembrane conductance regulator (CFTR) which encodes membrane bound chloride channel. The mutations prevent CFTR channel from operating properly and have been associated with symptoms ranging from mild to very severe pancreatic and lung disease. It is known that in the body CFTR protein is activated in a process called the cyclic AMP–dependent activation. The most common CFTR mutant, ΔF508CFTR, is poorly activated by this process. However, when the cyclic AMP–dependent pathway was activated in the presence of genistein, the activity of ΔF508CFTR was as large as that of normal CFTR (35). Thus, *in vitro*, genistein can restore the proper function of the mutant protein. Based on these data clinical trials of orally administrated genistein with cystic fibrosis patients are currently being planned.

The next possible application of genistein is the allergic diseases treatment. The underlying biochemical process implicated in this kind of diseases is mast cell degranulation in response to antigen challenge. Numerous factors released out of mast cell granules induce inflammatory reactions ranging from mild local urticaria to fatal anaphylaxis. In *in vitro* model of allergic asthma, genistein has been reported to block mast cell degranulation. It was reflected in the reduction of release of histamine and peptidoleukotrienes and in the decrease of anaphylactic contraction of the bronchi following antigen stimulation. In addition, genistein appeared to inhibit bronchial contraction induced directly by leukotriene D4 or histamine treatment. These data suggested genistein to be a useful compound in the allergic diseases such as bronchial asthma (14).

## 6. Conclusion

Through the many years since its discovery, genistein has not attracted much attention of the scientific community because its meaning was confined to an estrogenic isoflavone, that shows unfavorable properties in susceptible animal species. Nevertheless, in the course of time it has drastically changed and recently there has been a veritable explosion in the interest for genistein. Numerous studies has focused on the mechanisms by which genistein functions in animal

organism exhibiting its health beneficial effects. This interest yielded a number of target proteins proposed to be cellular mediators of genistein action. The most important seem to be PTKs, PI/PIP kinases, topo II and ERs. Since genistein is able to influence such diverse targets, it is not surprising why so various cell types are affected by this compound. A substantial number of genistein mediated effects may be explained by the inhibition of PTKs and PI/PIP kinases. These enzymes are involved in pathways of signal transduction which finally lead to stimulation of a cell to perform its physiological (or pathological) function. Since genistein can inhibit propagation of activating signals common for majority of cell types, it is no wonder why this compound affects numerous human cells including lymphocytes, platelets, mast cells, epithelium cells, osteoclasts, cancer cells etc. Thus, stimulated lymphocytes act to destroy certain antigens – genistein by inhibiting this process acts as an immunosuppressant, activated platelets aggregate – genistein attenuates aggregation, mast cells release inflammatory mediators from cytoplasmic granules – genistein stabilizes mast cells, osteoclasts resorb bone tissue – genistein reduces destroying osteoclast potency, epithelium cells produce new capillaries – genistein inhibits neoangiogenesis, cancer cells constantly proliferate – genistein inhibits cell multiplication. Multidirectional activity of genistein can also be partly explained by inhibition of topo II an enzyme implicated in proper DNA function. Moreover, in estrogen–responsive cells genistein certainly interacts with ERs and such interactions may modulate numerous events in cellular life, too. To summarize it can be seen that genistein–mediated effects observed in a cell culture or *in vivo* are most likely the sum of discrete interactions with at least several intracellular target proteins. Depending on the cell type or construction of the experimental model the meaning of respective interaction may be higher or lower but to understand and explain genistein activities well, all of them should be taken into consideration.

Despite the fact that *in vitro* studies have revealed many possible mechanisms of genistein action, sometimes to demonstrate its particular activity, the compound had to be employed in extremely high concentrations far above those needed to kill cancer cells cultured *in vitro* or above concentrations attainable *in vivo* in laboratory animals. Accordingly, a question arises whether results of such studies should be seriously taken into consideration when genistein ability to thwart animal or human cancers is being evaluated. Whether genistein could at multiple lower concentrations (of several μM/l in culture medium or *in vivo* in plasma) affect treated cells through all of its reported mechanisms. In other words, how to distinguish between processes that occur *in vivo* and those that can be generated *in vitro*. To answer these questions it should be noted that methods used to investigate biochemical processes have certain defined thresholds of analytical sensitivity. So it is obvious that some subtle changes, induced by low genistein concentrations, would go behind the ability of detection by a respective method. Therefore, to demonstrate certain genistein activity to a measurable extent, the compound had to be used at very high concentrations.

Accordingly, it is not excluded that, *in vivo*, the isoflavone affects cells through most of its discovered mechanisms, however, each possible mechanism contributes only to a certain extent to the overall effect. Based on this hypothesis it can be assumed that genistein mode of action in the cell is a sum of small changes induced by the drug in target proteins. Thus, the compound may simultaneously activate ERs (if present), reduce activity of PI/PIP kinases or of susceptible PTKs, cause little DNA damage by blocking topo II, and influence certain genes expression, protein synthesis etc. All these alterations, although slight but numerous constitute total genistein's impact on the live cell and consequently on the whole organism. In conclusion, there is not a single target protein or receptor that could mediate and account for genistein action inside cell. Conversely, this compound attacks different cellular proteins, modulates series of biochemical reaction cascades what subsequently produces pleiotropic effects in the cell. Such a multidirectional mode of action makes genistein to be a potentially useful compound in numerous human diseases. Nevertheless, the interest of investigators is mainly focused on its anticancer properties. At low nutritionally relevant concentrations genistein predominantly effects healthy, nontransformed cells or those from early stages of tumorigenesis. By modulating various events in normal cellular life the isoflavone can inhibit the process of cancer initiation or promotion. However, plasma genistein levels achievable with soy food feeding are unlikely to be sufficient to attenuate the growth of mature, established tumors by chemotherapeutic–like mechanisms. In such cases fortified preparations of pure genistein, administered at multiple higher dosages might be a helpful novel anticancer drug.

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